Communication to the Editor

Accumulation of Toxic Advanced Glycation End-Products Induces Cytotoxicity and Inflammation in Hepatocyte-Like Cells Differentiated from Human Induced Pluripotent Stem Cells

Chigusa Kikuchi, a,b,c Chiko Sakasai-Sakai, a,b,c Risa Okimura, b Hinako Tanaka, b Takanobu Takata, d Masayoshi Takeuchi, d and Tamihide Matsunaga a,b

a Department of Clinical Pharmacy, Graduate School of Pharmaceutical Sciences, Nagoya City University; 3–1 Tanabe-dori, Mizuho-ku, Nagoya 467–8603, Japan; 
b Educational Research Center for Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Nagoya City University; 3–1 Tanabe-dori, Mizuho-ku, Nagoya 467–8603, Japan; c Laboratory of Community Medicine, Showa Pharmaceutical University; 3–3165 Higashi-Tamagawa Gakuen, Machida, Tokyo 194–8543, Japan; and d Department of Advanced Medicine, Medical Research Institute, Kanazawa Medical University; 1–1 Daigaku, Uchinadamachi, Ishikawa 920–0293, Japan.

Received June 20, 2021; accepted July 19, 2021

Nonalcoholic steatohepatitis (NASH), the aggressive form of the most common chronic liver disease nonalcoholic fatty liver disease, is characterized by inflammation and damage in the liver. Although hepatocyte injury and cell death have been identified as cardinal pathological features of NASH, its pathogenesis has not yet been elucidated in detail. Immortalized cell lines and primary cultured cells have been used as in vitro models of NASH. However, these cells have several disadvantages, such as specialized characteristics by immortalization or limited growth potential. To overcome these difficulties and develop a strategy to analyze the pathology of NASH, we employed hepatocyte-like cells differentiated from human induced pluripotent stem cells (hiPSC-HLCs) as an in vitro model of NASH to clarify the intracellular effects posed to the indicated concentrations of GA were measured according to the manufacturer’s instructions. WST-8 assay kit (Dojindo Laboratories, Kumamoto, Japan) was employed.

MATERIALS AND METHODS

Reagents Aminoguanidine (AG) was purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). GA was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Differe ntiation of hiPSCs into Hepatocytes The hiPSC line Windy, derived from the human embryonic lung fibroblast cell line MRC-5, was provided by Umezawa et al. of the National Center for Child Health and Development. hiPSCs were maintained as previously reported. Immortalized cell lines and primary cultured cells have been used as in vitro models of NASH. 3) A previous study using a human HCC cell line and primary hepatocytes reported that the intracellular accumulation of TAGE was associated with hepatocyte cell death. 6) 8) In comparisons of these two types of cells, primary hepatocytes are considered to more accurately reflect in vivo conditions. However, disadvantages are associated with a more detailed molecular analysis using primary hepatocytes because they cannot be passaged and, thus, it is challenging to increase cell numbers. Investigation using hepatocyte-like cells (HLCs) differentiated from human induced pluripotent stem cells (hiPSCs) (hiPSC-HLCs) is expected as a model of human liver cells derived from non-cancer cells. The present study investigated the effects of TAGE on hiPSC-HLCs. The results obtained demonstrated for the first time the cytotoxic and inflammatory effects of the accumulation of TAGE in hiPSC-HLCs.

INTRODUCTION

The worldwide prevalence of nonalcoholic fatty liver disease (NAFLD) is rapidly increasing. Nonalcoholic steatohepatitis (NASH) is a severe form of NAFLD, and has the greatest potential to progress to advanced fibrosis and cirrhosis, which are risk factors for hepatocellular carcinoma (HCC). 1) The overall mortality rate in NASH was previously reported to be 25.56 per 1000 patient years, with a liver-specific mortality rate of 11.77 per 1000 patient years. 2) Therefore, elucidating the pathogenesis of NASH is an important issue that warrants further study.

Advanced glycation end-products (AGEs), which are formed by non-enzymatic reaction between proteins and reducing-sugars, have been identified as one of the causes of NASH. 3) Two pathways are known for AGEs: (1) extracellular AGEs stimulate intracellular signaling pathways via RAGE, a cell membrane receptor for AGEs, and (2) intracellular AGEs induce protein dysfunction. Among the various AGEs, AGEs derived from glyceraldehyde (GA), an intermediate of glucose/fructose metabolism, are toxic AGEs (TAGE) that have been shown to contribute to the development of NASH. 4) Immortalized cell lines and primary cultured cells have been used as in vitro models of NASH. 3) A previous study using a human HCC cell line and primary hepatocytes reported that the intracellular accumulation of TAGE was associated with hepatocyte cell death. 6) 8) In comparisons of these two types of cells, primary hepatocytes are considered to more accurately reflect in vivo conditions. However, disadvantages are associated with a more detailed molecular analysis using primary hepatocytes because they cannot be passaged and, thus, it is challenging to increase cell numbers. Investigation using hepatocyte-like cells (HLCs) differentiated from human induced pluripotent stem cells (hiPSCs) (hiPSC-HLCs) is expected as a model of human liver cells derived from non-cancer cells. The present study investigated the effects of TAGE on hiPSC-HLCs. The results obtained demonstrated for the first time the cytotoxic and inflammatory effects of the accumulation of TAGE in hiPSC-HLCs.

MATERIALS AND METHODS

Reagents Aminoguanidine (AG) was purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). GA was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Differe ntiation of hiPSCs into Hepatocytes The hiPSC line Windy, derived from the human embryonic lung fibroblast cell line MRC-5, was provided by Umezawa et al. of the National Center for Child Health and Development. hiPSCs were maintained as previously reported. 9) Undifferentiated hiPSCs were basically differentiated into hepatocytes according to the previously reported methods. 10) Undifferentiated hiPSCs were basically differentiated into hepatocytes according to the previously reported methods. 10)

Cell Viability Assay Cell viability was assessed using the WST-8 assay kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions.

Slot Blotting Total TAGE in hiPSC-HLC extracts exposed to the indicated concentrations of GA were measured by slot blotting. This analysis was conducted as previously described. 10)
Real-Time RT-PCR Analysis  RNA extraction, a reverse transcription reaction, and real-time PCR analysis were performed as previously described. The housekeeping gene, hypoxanthine phosphoribosyltransferase (HPRT) was used as the normalization standard for mRNA expression levels. The primers used in this experiment are listed in Table 1.

Table 1. Sequences of Primers for the Real-Time RT-PCR Analysis

| Gene   | Forward primer                      | Reverse primer                      |
|--------|-------------------------------------|-------------------------------------|
| IL-6   | 5'-AGCCACTCACCTTTCAGAAG-3'          | 5'-GCCTTTGCTGGTTCCACAC-3'           |
| IL-8   | 5'-CTGATTITCGACGTTGCTTG-3'          | 5'-GGTTGGAAAGTTGGAGGTATAG-3'        |
| MCP-1  | 5'-TCCCAAGAGCTGATCTTCTCA-3'         | 5'-TGCTTGCCAGGTGTCCCAT-3'           |
| HPRT   | 5'-CTTTGCTTTCCTGGTCAGG-3'           | 5'-TCAAGGGAATCCACATTACACA-3'        |

The abbreviations used are as follows: IL-6; interleukin-6, IL-8; interleukin-8, MCP-1; monocyte chemoattractant protein-1, HPRT; hypoxanthine phosphoribosyltransferase.

RESULTS

The GA Treatment Induces the Accumulation of TAGE and Cytotoxicity in hiPSC-HLCs  Previous studies reported the accumulation of TAGE in HCC cell lines, including HepG2 and Hep3B, treated with extracellular GA. To establish whether TAGE also accumulate in hiPSC-HLCs, hiPSC-HLCs were treated with GA and cell extracts were analyzed with anti-TAGE antibody. The results obtained showed that the accumulation of TAGE was significantly increased by the treatment with 4 mM GA (Fig. 1a). Furthermore, a preincubation with AG, an inhibitor of AGE formation, suppressed the accumulation of TAGE (Fig. 1b). We then assessed cytotoxicity based on the accumulation of TAGE. The cell viability of hiPSC-HLCs was significantly reduced at a GA concentration of 4 mM (Fig. 1c).

The GA Treatment Increases Intracellular Inflammation Pathway in hiPSC-HLCs  To confirm whether the accumulation of TAGE caused inflammation in hiPSC-HLCs, the expression of inflammation markers, including interleukin 6 (IL-6), IL-8, and monocyte chemoattractant protein-1 (MCP-1), was quantified using real-time RT-PCR. The results obtained showed that the mRNA expression levels of IL-6, IL-8, and MCP-1 increased in a GA dose-dependent manner (Fig. 2).

DISCUSSION

AGEs that accumulate in the liver, the key organ of excess sugar metabolism, are involved in the pathology of NASH. In NASH patients, the accumulation of TAGE was shown to strongly correlate with disease onset and progression. TAGE accumulation has been reported to induce damage to primary hepatocytes and HCC cell lines, HepG2 and Hep3B. We previously showed that the intracellular accumulation of TAGE induced by GA promoted the disruption of the apoptotic pathway and the production of reactive oxygen species, and decreased chaperone activity in HCC cell lines, and the
mechanisms underlying TAGE-induced cytotoxicity are being extensively examined.6–8) In the present study, we investigated whether hiPSC-HLCs may be used to analyze TAGE toxicity because they are expected as a model of human liver cells derived from non-cancer cells. We examined hiPSC-HLC cell death induced by the accumulation of TAGE and its role on the expression of inflammatory markers. We used a millimolar concentration of GA to induce TAGE accumulation in cells because we previously reported that the same TAGE-modified proteins were detected in Hep3B cells cultured for 5 d with a high fructose concentration and cultured for 6 h with 4 mM GA.12) Therefore, a millimolar concentration of GA is optimal under experimental conditions conducted for a short time period. Regarding TAGE accumulation, the GA treatment induced the formation of TAGE in hiPSC-HLCs (Fig. 1a) as well as in HepG2 and Hep3B as previously reported.6–8) Regarding the amount of TAGE that accumulated following a treatment with 4 mM GA, TAGE levels were lower in hiPSC-HLCs than in HepG2 and Hep3B cells. However, cell viability assessed using the WST assay was similar between Hep3B cells and hiPSC-HLCs (Fig. 1c). The reason why less TAGE accumulated in hiPSC-HLCs than in Hep3B regardless of similar survival rates currently remains unclear. Differences in cellular sensitivity to the accumulation of TAGE may indicate that the target proteins for TAGE modifications and its function in survival differ between these two cell types. Further studies are needed to clarify the different mechanisms in these cells. Cell death was not induced when TAGE levels of approximately 2 µg/mg protein accumulated in hiPSC-HLCs (Fig. 1). However, TAGE levels of more than 5 µg/mg protein induced cell death in hiPSC-HLCs and Hep3B cells.6) Regarding the liver, we previously showed that rats fed a high fructose diet had TAGE levels of approximately 3 µg/mg protein in the liver, and liver damage had not yet been detected in this state.11) These findings suggest that TAGE levels of approximately 2 to 3 µg/mg protein do not induce liver damage, whereas cellular damage is detected from TAGE levels of approximately 5 µg/mg protein.

In addition to hepatocyte cell death, the inflammation of hepatocytes is a prominent feature of NASH. The mRNA expression level of C-reactive protein in Hep3B cells was increased by GA treatment, indicating TAGE-induced inflammatory effects.6) The inflammatory effects of the GA treatment in hiPSC-HLCs were investigated in the present study. We employed inflammation markers, including IL-6, IL-8, and MCP-1, which are proinflammatory cytokines related to the progression of NAFLD.13) The mRNA expression levels of these markers were increased in hiPSC-HLCs by the treatment with GA (Fig. 2). In addition to inflammation, oxidative stress and other phenotypes of NASH need to be analyzed in hiPSC-HLCs in the future.

In the present study, we examined the effect of TAGE accumulation in hiPSC-HLCs and compared its effect with the result of HCC cell lines. TAGE accumulation and inflammatory effects were similar tendency in GA-treated hiPSC-HLCs and HCC cell lines. Experimental data from hiPSC-HLCs are considered to more accurately reflect the pathology of humans; however, marked differences were not detected in the present study. If differences exist between hiPSC-HLCs and HCC cell lines, data obtained from hiPSC-HLCs may be a stepping stone to more accurately reflect in vivo conditions. At this stage, the combined use of hiPSC-HLCs and HCC cell lines, data obtained from hiPSC-HLCs may be a stepping stone to more accurately reflect in vivo conditions. The present study provides a novel strategy for examining the involvement of hepatocyte toxicity due to accumulation of TAGE in the pathogenesis of NASH.

**Acknowledgments** This work was supported by JSPS KAKENHI Grant Nos. 16H01811 (to Takeuchi) and 19H03391 (to Matsunaga).

**Conflict of Interest** The authors declare no conflict of interest.
REFERENCES

1) Takahashi Y, Fukusato T. Histopathology of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World J. Gastroenterol., 20, 15539–15548 (2014).

2) Povsic M, Wong OY, Perry R, Bottomley J. A structured literature review of the epidemiology and disease burden of non-alcoholic steatohepatitis (NASH). Adv. Ther., 36, 1574–1594 (2019).

3) Rungratanawanich W, Qu Y, Wang X, Essa MM, Song BJ. Advanced glycation end products (AGEs) and other adducts in aging-related diseases and alcohol-mediated tissue injury. Exp. Mol. Med., 53, 168–188 (2021).

4) Sakasai-Sakai A, Takata T, Takino J, Takeuchi M. The relevance of toxic AGEs (TAGE) cytotoxicity to NASH pathogenesis: a mini-review. Nutrients, 11, 462 (2019).

5) Chavez-Tapia NC, Rosso N, Tiribelli C. In vitro models for the study of non-alcoholic fatty liver disease. Curr. Med. Chem., 18, 1079–1084 (2011).

6) Takino J, Kobayashi Y, Takeuchi M. The formation of intracellular glyceraldehyde-derived advanced glycation end-products and cytotoxicity. J. Gastroenterol., 45, 646–655 (2010).

7) Sakasai-Sakai A, Takata T, Takino J, Takeuchi M. Impact of intracellular glyceraldehyde-derived advanced glycation end-products on human hepatocyte cell death. Sci. Rep., 7, 14282 (2017).

8) Sakasai-Sakai A, Takata T, Takeuchi M. Intraacellular toxic advanced glycation end-products promote the production of reactive oxygen species in HepG2 cells. Int. J. Mol. Sci., 21, 4861 (2020).

9) Iwao T, Toyota M, Miyagawa Y, Okita H, Kiyokawa N, Akutsu H, Umezawa A, Nagata K, Matsunaga T. Differentiation of human induced pluripotent stem cells into functional enteroocyte-like cells using a simple method. Drug Metab. Pharmacokinet., 29, 44–51 (2014).

10) Kondo Y, Iwao T, Yoshiihashi S, Mimori K, Ogihara R, Nagata K, Kurose M, Niwa T, Suzuki T, Miyata N, Ohmori S, Nakamura K, Matsunaga T. Histone deacetylase inhibitor valproic acid promotes the differentiation of human induced pluripotent stem cells into hepatocyte-like cells. PLOS ONE, 9, e104010 (2014).

11) Takata T, Sakasai-Sakai A, Takino J, Takeuchi M. Evidence for toxic advanced glycation end-products generated in the normal rat liver. Nutrients, 11, 1612 (2019).

12) Takino J, Nagamine K, Takeuchi M, Hori T. In vitro identification of nonalcoholic fatty liver disease-related protein hnRNPM. World J. Gastroenterol., 21, 1784–1793 (2015).

13) Braundersreuther V, Vivanti GL, Mach F, Montecucco F. Role of cytokines and chemokines in non-alcoholic fatty liver disease. World J. Gastroenterol., 18, 727–735 (2012).