Advanced glycation end-products (AGEs) are produced by the non-enzymatic reaction of sugars with proteins. It has been revealed that glyceraldehyde-derived toxic AGEs (TAGE) are elevated in the serum of non-alcoholic steatohepatitis (NASH) patients. NASH causes liver fibrosis and progresses to cirrhosis and hepatocellular carcinoma. However, the impact of TAGE in liver fibrosis caused by extracellular matrix accumulation remains poorly understood. In this study, we examined the effect of TAGE on the activation of hepatic stellate cells that are involved in liver fibrosis. LX-2 cells treated with transforming growth factor-β1 (TGF-β1) significantly reduced cell viability by apoptosis. However, the decrease in cell viability with TGF-β1 treatment was significantly suppressed by TAGE co-treatment. The levels of α-smooth muscle actin (α-SMA) and platelet-derived growth factor (PDGF)-Rβ and its ligand PDGF-B were increased in LX-2 cells following TGF-β1 treatment, suggesting that these cells were activated; however, these increases were unaffected by TAGE co-treatment. Moreover, collagen I level was increased with TGF-β1 treatment, and this increase was further increased by TAGE co-treatment. These results suggest that the suppression of apoptosis in activated LX-2 cells by TGF-β1 and TAGE co-treatment is related to an increase in the production of the extracellular matrix such as collagen I. Therefore, it was suggested that TAGE might aggravate the liver fibrosis of chronic hepatitis, such as NASH.

Key words glycation; non-alcoholic steatohepatitis; liver fibrosis; transforming growth factor-β1

INTRODUCTION

Liver fibrosis is characterized by the accumulation of extracellular matrix (ECM) caused by chronic liver injury including non-alcoholic steatohepatitis (NASH), and its progression causes liver cirrhosis.1) NASH, a liver disease of metabolic syndrome that is characterized by insulin resistance, diabetes mellitus, and obesity, is a hepatitis with histopathological findings of alcoholic liver disease that occurs in patients without alcoholism.2–5) Approximately 8 to 26% of NASH patients progress to cirrhosis, and about 10% of NASH cirrhosis patients develop hepatocellular carcinoma (HCC) after 5 years.5,7)

Hepatic stellate cells (HSCs) that localize to the space of Disse, between hepatocytes and sinusoidal endothelial cells, are the major fibrogenic cells of liver fibrosis.8,9) Under normal conditions, HSCs are quiescent, and their main function is vitamin A storage.8) On the other hand, in chronic liver injury, HSCs become active through various cytokines such as tumor necrosis factor-α, platelet-derived growth factors (PDGFs), and transforming growth factor-β1 (TGF-β1), resulting in differentiation into myofibroblast-like cells and secretion of a large amount of ECM, such as collagen I.9) TGF-β1 is a significant activator of HSCs.10) Furthermore, activation of HSCs also participates in the onset and progression of HCC.11–13)

The reactivity of the advanced glycation end-products (AGEs) produced by the non-enzymatic reaction between sugar and protein depends on the type of sugar or carbonyl compound.14) Indeed, evidence suggests that among the various AGEs, glyceraldehyde-derived toxic AGEs (TAGE) closely participate in diabetic complications, insulin resistance, hypertension, obesity, cancer, dementia, cardiovascular diseases, and NASH.15–23) We recently found that serum TAGE levels were significantly higher in NASH patients than in healthy controls and simple steatosis patients, and TAGE was accumulated in the livers of NASH patients.24) Furthermore, we recently demonstrated that serum TAGE levels were significantly decreased with the improvement of NASH by treatment with atorvastatin, a hydroxymethylglutaryl-CoA reductase inhibitor, in patients with NASH and dyslipidemia.25) Therefore, TAGE may have some effect on NASH-associated liver fibrosis associated.

In the present study, we examined the effects of TAGE on TGF-β1-activated HSCs using human HSCs, i.e., LX-2 cells, and showed that TAGE increases the total production of ECM by suppressing cell death in TGF-β1-activated HSCs.

MATERIALS AND METHODS

Reagents TGF-β1 was purchased from ProSpec-Tany TechnoGene Ltd. (Ness-Ziona, Israel). TAGE and control bovine serum albumin (BSA) were prepared as previously described.26) Q-VD-OPH was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A). All reagents marketed in high purity were used as supplied.

Cell Cultures LX-2 cells were grown in high glucose me-
dium (Dulbecco’s modified Eagle’s medium (DMEM); Sigma-Aldrich, St. Louis, MO, U.S.A.) added with 2% fetal bovine serum (Equitech-Bio, Kerrville, TX, U.S.A.). Cells were seeded in culture dishes or plates and incubated for 24 h prior to use in experiments. Cells were incubated with or without TGF-β1 (2.5 ng/mL) for 6–120 h in serum-free DMEM.

**Cell Viability and Cell Proliferation** It was performed as previously described.27) The net difference in absorbance (A450–A650) was used as a measure of cell viability and cell proliferation. Viability of BSA-treated cells or 0 h was taken as 100%.

**Preparation of Cell Lysate and Western Blot** They were performed as previously described,27) using the following antibodies: rabbit anti-poly(ADP-ribose)polymerase (PARP) antibody (GeneTex, Alton Pkwy Irvine, CA, U.S.A., GTX100573 at 1:1500), mouse anti-β-actin antibody (Santa Cruz Biotechnology, Dallas, TX, U.S.A., sc-47778 at 1:12000), mouse anti-α-actin antibody (Santa Cruz Biotechnology, sc-32251 at 1:2000), anti-rabbit immunoglobulin G (IgG) antibody (GeneTex, GTX77057 at 1:5000), and anti-mouse IgG antibody (Dako Denmark A/S, Glostrup, Denmark, P0260 at 1:5000).

**Real-Time RT-PCR** It was performed as previously described,28) and the primers used were as follows: α-smooth muscle actin (α-SMA), 5'-ACT GGG ACG ACA TGG AAA AG-3' and 5'-TAG ATG GGG ACA TTT TGG GTG-3'; PDGF-Rβ, 5'-ATG CCT TAC CAC ATC CGC TC-3' and 5'-ACA TGG CAG GTG TAG GTG CCC-3'; PDGF-B, 5'-AGG AGA CCC TTG GAG CCT AG-3' and 5'-CAT CGA GAC AGA CGG ACG AG-3'; collagen type I alpha 1 (COL1A1), 5'-ACC TGG TCA AAC TGG TCC TG-3' and 5'-ACC TCC CAC TCC AGC AGA TGT GG-3'; and β-actin, 5'-TCC ACC TCC AGC AGA TGT GG-3' and 5'-GCA TTT GCG GTG GAC CAT-3'.

**Enzyme-Linked Immunosorbent Assay (ELISA)** The conditioned medium was collected and used with a Human Pro-Collagen I alpha 1 ELISA kit (abcam, Cambridge, UK, ab210966). All processes were performed according to the manufacturer’s instructions.

**Statistical Analysis** It was performed as previously described.27)

### RESULTS

**TAGE Suppresses TGF-β1-Induced Decrease of Cell Viability in LX-2 Cells** To investigate the effect of TAGE on activated LX-2 cells, we examined the cell viability of LX-2 cells in the treatment of an unglycated control BSA and TAGE with or without TGF-β1. Cell viability of LX-2 cells was decreased with TGF-β1 treatment in the presence of BSA at any concentration (5, 10, 20, and 40 µg/mL). However, decreasing the cell viability with TGF-β1 treatment was significantly suppressed in a concentration-dependent manner with TAGE (Fig. 1a). There was no change when the concentration of TAGE was raised to 40 µg/mL. This result suggested that the inhibitory effect is maximized at a concentration of 20 µg/mL, and subsequent experiments were performed at a TAGE concentration of 20 µg/mL.

As shown in Fig. 1b, when the cell proliferation rate at 0 h was 100%, the cell proliferation with BSA and TAGE treatment increased in a time-dependent manner. TAGE treatment increased cell proliferation for 120 h compared to BSA treatment. On the other hand, co-treatment with TGF-β1 significantly suppressed cell proliferation, but TAGE and TGF-β1 co-treatment significantly restored cell proliferation compared to BSA and TGF-β1 co-treatment. The difference was greater in a time-dependent manner.

**TAGE Suppresses TGF-β1-Induced Apoptosis in LX-2 Cells** We examined whether the recovery of the cell viability by TAGE is due to the suppression of cell death by apoptosis. The expression of cleaved PARP, which is a marker of apoptosis, was undetected in each treatment alone and TAGE and TGF-β1 co-treatment for 6 h but was only detected by BSA and TGF-β1 co-treatment (Fig. 2a). The cleaved PARP was detected by co-treatment with TGF-β1 for 12 h, but TAGE and TGF-β1 co-treatment was less than BSA and TGF-β1 co-treatment (Fig. 2b). The decrease in cell viability with TGF-β1 treatment was significantly restored by pre-treatment with Q-VD-OPH, which is a caspase inhibitor (Fig. 2c). The recovery rate in Q-VD-OPH pre-treatment was clearly higher in BSA and TGF-β1 co-treatment. Cell viabilities in the treatment of BSA and TAGE were also significantly increased by Q-VD-OPH pre-treatment. These results indicate that TAGE
suppresses TGF-β1-induced apoptosis.

**TAGE Did Not Interfere with the Activation of LX-2 Cells by TGF-β1 Treatment** The activation of HSCs starts by transcription of target genes from the TGF-β1 signaling pathway. To determine whether TAGE interfered with the activation of LX-2 cells by TGF-β1 treatment, we confirmed the expression of α-SMA and PDGF-Rβ and its ligand PDGF-B. The mRNA expression of α-SMA, a marker of HSC activation, was unchanged by BSA and TAGE treatment, whereas the expression increased about 2.5-fold by co-treatment with TGF-β1. There was no significant difference between BSA and TAGE in the presence of TGF-β1 (Fig. 3c). Similarly, the protein expression of α-SMA was unchanged by BSA and TAGE treatment but was increased by co-treatment with TGF-β1. There was no difference between BSA and TAGE in the presence of TGF-β1 (Fig. 3b).

In addition, the mRNA expression of PDGF-Rβ and its ligand PDGF-B, which are expressed in an HSC activation-dependent manner, increased by about 2.5-fold and 3-fold, respectively, by co-treatment with TGF-β1. There was no significant difference between BSA and TAGE in the presence of TGF-β1 (Figs. 3c, d). Therefore, TAGE did not interfere with the activation of LX-2 cells by TGF-β1 treatment.

**TAGE Further Increased Collagen I Protein Production with TGF-β1 Treatment in LX-2 Cells** Myofibroblast-like differentiated activated HSCs produce excessive amounts of ECM, leading to further progression of liver fibrosis. To determine whether the fibrotic ability by HSC activation was altered by TAGE, the collagen I mRNA expression of the LX-2 cells and its protein concentration in the culture medium were measured. The expression of the collagen I mRNA was unchanged by BSA and TAGE treatment but was increased by about 4-fold by co-treatment with TGF-β1. There was no significant difference between BSA and TAGE in the presence of TGF-β1 (Fig. 4a). On the other hand, the concentration of collagen I protein, which was secreted from LX-2 cells in the
culture medium, was unchanged by BSA and TAGE treatment but was significantly increased by co-treatment with TGF-β1. Moreover, the concentration of collagen I protein of TAGE and TGF-β1 co-treatment was significantly higher than that of BSA and TGF-β1 co-treatment (Fig. 4b).

**DISCUSSION**

We previously reported on the formation of TAGE and its serum concentration in liver diseases, and the clinical data were expressed as arbitrary units (U), 1U corresponds to 1 µg of standard TAGE by the reaction of glyceraldehyde and BSA.24,25,30 The formation of TAGE was shown in the livers of NASH patients by immunohistochemistry, and serum TAGE levels were significantly higher in NASH patients (9.73 ± 3.73 U/mL n = 66) than in healthy controls and simple steatosis patients.24 Furthermore, serum TAGE levels in patients with NASH and dyslipidemia (10.4 ± 3.8 U/mL n = 43) were also significantly higher than 6 or 12 months after the treatment of atorvastatin.25 In addition, serum TAGE levels were significantly higher in non-B or non-C HCC patients (correction value, 18.2 ± 5.4 U/mL n = 90) than in healthy controls and NASH patients without HCC.30 Additionally, the higher TAGE levels in the serum of these patients reached about 25 U/mL. In this study, the decrease in cell viability of activated HSCs, i.e., LX-2 cells, with TGF-β1 treatment was significantly suppressed by TAGE treatment (<20 µg/mL) (Figs. 1a, b). Therefore, the concentration of TAGE (20 µg/mL) used in this study was consistent with the clinical data.

Liver fibrosis is caused as a result of ECM accumulation by
HSC activation to differentiate into myofibroblast-like cells.\textsuperscript{9} The amount of ECM produced is determined by the matrix production capacity per cell and the number of cells.\textsuperscript{31} In HSCs, TGF-β1 is an activator and the most potent pro-fibrotic cytokine that stimulates the production of various extracellular matrices at the gene level.\textsuperscript{10} On the other hand, TGF-β1 is also known to suppress proliferation and induce apoptosis in various cells.\textsuperscript{32} Recently, Tee et al. reported that LX-2 cells induced apoptosis while being activated by TGF-β1 treatment.\textsuperscript{33} Indeed, we also demonstrated that treatment of TGF-β1 induced apoptosis in the LX-2 cells, and it was significantly restored by Q-VD-OPH pre-treatment (Figs. 2a, b). The suppression of apoptosis-derived cell death by TGF-β1 treatment in activated HSCs might further promote fibrosis in the liver.

TGF-β1-induced apoptosis was suppressed in the presence of TAGE (Fig. 2a). Although the expressions of α-SMA, an indicator of HSC activation, increased through TGF-β1, the expression level is unaffected by TAGE co-treatment (Figs. 3a, b). Similarly, the expressions of PDGF-Rβ and its ligand PDGF-B, which were also involved in HSC activation and its proliferation, with TGF-β1 treatment were unaffected by TAGE co-treatment (Figs. 3c, d). These results suggest that TAGE suppresses apoptosis without interfering with the activation of HSC by TGF-β1 treatment. Moreover, the expression level of mRNA of collagen I per cell was unchanged, whereas the amount of collagen I protein secreted in the culture medium was significantly increased in TAGE and TGF-β1 co-treatment (Figs. 4a, b). These suggested that the increase of collagen I protein was due to the number of LX-2 cells, not to the ability of collagen I production per cell, because TAGE suppressed TGF-β1-induced apoptosis. Therefore, other ECM proteins, in addition to collagen I, are also increasing.

It is known that the gene expressions of α-SMA and collagen I with TGF-β1 treatment are mediated via the SMAD pathway, whereas apoptosis by TGF-β1 treatment is also mediated via the non-SMAD pathway.\textsuperscript{10,32,34} Therefore, it was suggested that TAGE may suppress TGF-β1 signaling via the non-SMAD pathway or may be involved in pathways other than TGF-β1 signaling.

In summary, we demonstrated that TAGE increased the total production of ECM, such as collagen I, by suppressing apoptosis in activated HSCs, i.e., LX-2 cells, following TGF-β1 treatment. Therefore, it was suggested that TAGE might aggravate the liver fibrosis of chronic hepatitis, such as NASH.

Acknowledgments This work was supported by JSPS KAKENHI Grant Nos. JP16H01811 (for Takeuchi) and JP18K11036 (for Hori).

Conflict of Interest The authors declare no conflict of interest.

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