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ORIGINAL CONTRIBUTION

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Effects of high-AGE beverage on RAGE and VEGF expressions in the liver and kidneys

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Abstract *Background* The formation and accumulation of advanced glycation end products (AGEs) increase in some lifestyle-related diseases as well as in aging; however, little is known about the relationship between food-derived AGEs and the pathology of such diseases. *Aim of the study and methods* To explore whether food items containing high levels of AGEs are involved in the development of lifestyle-related diseases, rats were orally administered a commercial high-AGE beverage [*Lactobacillus* beverage-A (LB-A)]. With a particular focus on angiogenesis-associated diseases, the gene expressions of vascular endothelial growth factor (VEGF) and the receptor for AGEs (RAGE) were examined in the liver and kidneys using real-time reverse transcription-polymerase chain reaction. Moreover, AGE deposition was immunohistochemically investigated in these tissues. *Results and conclusions* Hepatic VEGF expression was significantly increased in rats administered LB-A ($P < 0.01$ vs. control). Furthermore, immunohistochemical analysis detected glucose-derived AGE-positive cells in the liver from the LB-A group. These results suggest that AGE-rich beverages increase hepatic VEGF expression and AGE accumulation, bringing about early events associated with lifestyle-related diseases.

Key words *Lactobacillus* beverage – food-derived AGEs – lifestyle-related diseases – VEGF – RAGE

Introduction

Advanced glycation end products (AGEs) are protein adducts produced from lipids, nucleic acids, glucose, α-hydroxyaldehydes (glyceraldehyde and glycolaldehyde), and dicarbonyl compounds (glyoxal, methylglyoxal, and 3-deoxyglucosone) through non-enzymatic reactions. Chronic hyperglycemia, a major characteristic of diabetes mellitus, has been considered to accelerate the formation of AGEs in various tissues. AGEs, thus generated, can be involved in the onset of lifestyle-related disease [1, 16], in which they induce the expression of several genes, including that for vascular endothelial growth factor (VEGF), after binding to the specific receptor (RAGE) [22, 23]. VEGF is known to play a critical role in angiogenesis, and excessive angiogenesis is one of the major vascular changes implicated in diseases such as cancer and diabetic retinopathy [6].

Although many reports have shown that endogenous AGEs are involved in the pathogenesis of these diseases, little is known about the implication of food-derived AGEs in pathogenic processes, including

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vascular changes. These exogenous AGEs can be formed during high-heat cooking and food processing, including fermentation. We measured AGE content in various commercial products by ELISA and found that *Lactobacillus* beverage-A (LB-A) contains high concentrations of glucose-derived AGEs. To obtain more information about the relationship between a high-AGE beverage and the pathogenic processes of lifestyle-related diseases, especially those of angiogenesis-associated diseases, LB-A was orally administered to rats, and the gene expressions of VEGF and RAGE were investigated in the liver and kidneys followed by immunohistochemical analysis to determine AGE accumulation in these tissues.

Materials and methods

Materials

Exscript RT reagent kit and SYBR Premix Ex *Taq* reagent were purchased from Takara Bio Inc. (Shiga, Japan). Immunostaining biotin-streptavidin (LSAB2) kit was purchased from Dako (CA, USA). Rat Insulin ELISA kit was purchased from Shibayagi Co. Ltd (Gunma, Japan). ISOGEN was obtained from Nippon Gene (Tokyo, Japan). Other reagents were purchased from Wako Chemicals (Osaka, Japan). Polyclonal anti-glucose-derived AGE antibody was raised as described in the previous paper [18]. The major components of LB-A are protein (12.3 mg/ml), lipid (1.54 mg/ml), carbohydrate (177 mg/ml), and *Lactobacillus* (2.31×10^8 cfu/ml), providing a total energy of 0.77 kcal/ml. Glucose-derived AGE levels in LB-A and other beverages were measured by ELISA and the following values were obtained: LB-A, 5,400 U/ml; cola, 110 U/ml; vegetable juice, 80 U/ml; and green tea, 0 U/ml [one unit (U) represents 1 μg of glucose-derived AGE-bovine serum albumin], indicating that LB-A contained high concentrations of glucose-derived AGEs compared to other beverages.

Animals and experimental designs

All animal experiments followed the Guidelines for the Proper Conduct of Animal Experiments established by the Science Council of Japan (1 June 2006). Male Sprague-Dawley rats aged seven weeks were maintained under standard temperature and humidity conditions on a 12-h light/dark cycle with free access to standard chow diet and tap water. Animals were observed once daily for general appearance and signs of ill health during the experimental period. Rats were orally administered either 2 ml/day of LB-A ($n = 6$) or distilled water (control, $n = 6$) through a stainless steel feeding tube attached to a 2.5-ml syringe once daily for eight weeks. During the administration period, body weight was measured twice a week, and food and water intake were monitored once a week.

After 8 weeks of administration, blood was sampled from the inferior vena cava under anesthesia. Serum glucose, insulin, and glucose-derived AGE levels were measured by a glucose test kit, the rat Insulin ELISA kit, and competitive ELISA [18, 19], respectively. Dissected liver and kidney were immediately frozen in liquid nitrogen and stored at −80°C until RNA isolation. For immunohistochemical analysis, aliquots of liver and kidney tissues were fixed in 10% formalin followed by a standard paraffin-embedding procedure.

Isolation of total RNA from tissues

Total RNA was isolated from liver and kidney samples using a commercial reagent (ISOGEN) according to the manufacturer's procedures. In brief, tissues were homogenized with a Potter glass homogenizer for ten strokes and mixed with chloroform. After centrifugation at 4°C for 15 min (12,000×*g*), the aqueous phase was transferred into a new plastic tube, mixed with isopropanol, centrifuged again under the above conditions for 10 min, and washed with 70% ethanol. The precipitate was dried and dissolved in 50 μl of sterilized water. All samples were stored at −80°C.

Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR)

Complementary DNA was synthesized with the Exscript RT reagent kit. Real-time RT-PCR was performed using the Smart Cycler II system (Cepheid, CA, USA) and SYBR Premix Ex *Taq* reagent. *β*-actin was used as an internal control. Primers designed to produce mRNA-specific amplification products were as follows: *β*-actin, 5′-GCC CTG GCT CCT AGC ACC-3′, and 5′-CCA CCA ATC CAC ACA GAG TAC TTG-3′; VEGF, 5′-GGT CCC AGG CTG CAC CCA CG-3′, and 5′-TTA GGG GCA CAC AGG ACG GC-3′; and RAGE, 5′-CAG GGT CAC AGA AAC CGG-3′, and 5′-ATT CAG CTC TGC ACG TTC CT-3′.

Immunohistochemistry

Formalin-fixed, paraffin-embedded liver and kidney tissue sections (5 μm) were immunohistochemically stained for glucose-derived AGEs using the LSAB2 kit. Immunoreactivity with polyclonal anti-glucose-derived AGE antibody [18] was visualized with 3-3′-

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diaminobenzidine and counterstained with Mayer's hematoxylin.

Statistical analysis

Values are expressed as the mean ± SD. Statistical differences were determined by the Wilcoxon rank sum test, and P values less than 0.05 were considered significant.

Results

Body weight, water and food intake, and blood biochemistry

Body weight gain was similar in both the LB-A and control groups, and food and water intake was stable during the experimental period. No differences were found between the two groups regarding serum glucose, insulin, and glucose-derived AGE levels (data not shown).

Quantitative analysis of VEGF and RAGE expression in the liver and kidneys

Liver and kidney weights were not different between the LB-A and control groups. Real-time RT-PCR was performed to quantify the gene expression of VEGF and RAGE in the liver and kidneys. Hepatic VEGF expression was significantly increased in the LB-A compared to the control ($P < 0.01$, Fig. 1A). Although statistically not significant, RAGE expression was also increased in the liver (Fig. 1B). In the kidneys, VEGF and RAGE expressions were not different between the two groups (Fig. 1C, D).

Immunohistochemistry

Glucose-derived AGE-immunopositive granules were detected only in the liver from LB-A, suggesting hepatic AGE accumulation (Fig. 2). Kidney tissues from both groups did not show any differences in immunoreactivity to AGEs (data not shown).

Discussion

The involvement of AGEs in the pathogenesis of lifestyle-related diseases has been investigated well over the last two decades; however, limited information is available on whether and how food-derived AGEs are implicated in these diseases. Continuous intake of a high-AGE diet has been shown to increase serum AGE levels [9, 10]. Reports have also demonstrated in apolipoprotein-E deficient mice that a low-AGE diet reduced neointimal formation after femoral artery injury [9] and prevented atherosclerotic lesion

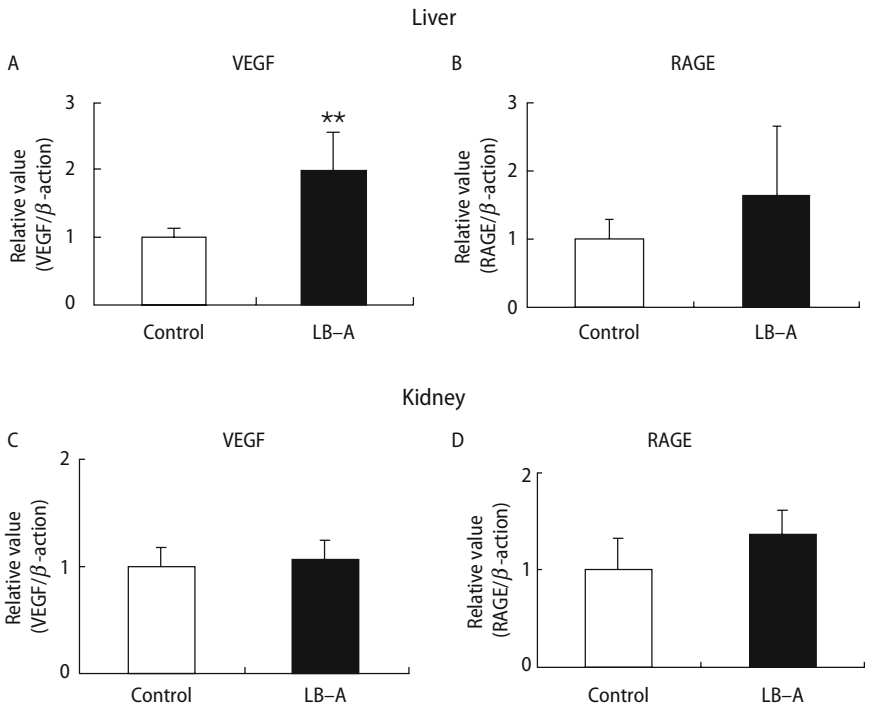

Fig. 1 Expression of vascular endothelial growth factor (VEGF) and receptor for advanced glycation end products (RAGE) in the liver (**A** and **B**, respectively) and kidneys (**C** and **D**, respectively) in rats administered *Lactobacillus* beverage-A (LB-A, *closed bars*) and distilled water (control, *open bars*) for 8 weeks. Total RNAs isolated from the liver and kidneys were used for real-time reverse transcription-PCR. Data are expressed as relative values of control. $^{**}P < 0.01$ versus control, mean ± SD ($n = 6$)

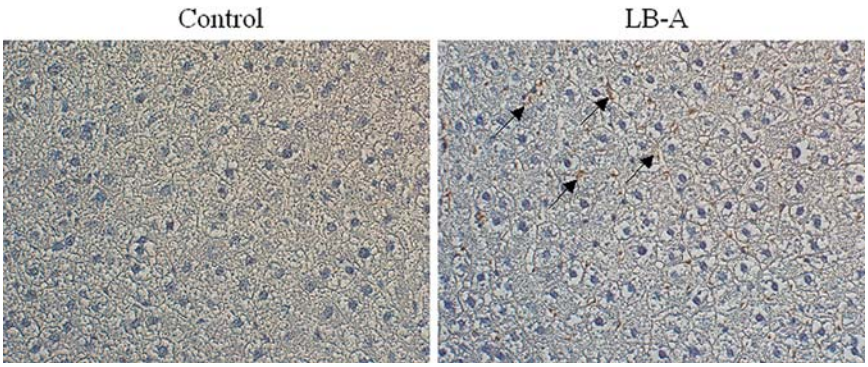

Fig. 2 Immunohistochemical staining for glucose-derived advanced glycation end products (AGEs) in the liver from rats administered *Lactobacillus* beverage-A (LB-A) and distilled water (control) for eight weeks. Immunoreactivity with polyclonal anti-glucose-derived AGEs antibody was visualized with 3-3′-diaminobenzidine (brown color) and counterstained with Mayer's hematoxylin. Arrows indicate glucose-derived AGE-positive granules (×400)

development after inducing streptozotocin diabetes [10]. In diabetic subjects, a high-AGE diet increased serum levels of inflammatory mediators associated with tissue injury, such as mononuclear cell tumor necrosis factor-α [21]. Modifying dietary AGE intake can provide preventive and therapeutic effects against lifestyle-related vascular changes. Goldberg et al. [5] demonstrated AGE content in several food items as follows: butter, 1,324 kU/5 g serving; roasted beef, 5,464 kU/90 g serving; processed cheese, 2,603 kU/30 g serving; egg yolk (boiled), 182 kU/30 g serving; and tofu (raw), 709 kU/90 g serving, and suggested that diet can be a significant environmental source of AGEs, which may constitute a chronic risk factor for cardiovascular and kidney damage. In previous studies, however, subjects and animals received an AGE-enriched diet, not a food item containing high levels of AGEs. To the best of our knowledge, this report is the first to use a commercial product as a source of AGEs and to observe the possible involvement of an AGE-rich food item in the pathogenic processes of lifestyle-related diseases.

Several reports have demonstrated that AGEs induced the expression of VEGF, a positive regulator of vascular formation and angiogenesis [6]. VEGF expression was increased in the eyes when mice were injected intraperitoneally with AGEs [20], and similar effects were observed in cells cultured in media supplemented with AGEs [13, 22, 23]. VEGF gene overexpression is known to cause hepatic fibrosis [12, 24], the pathology of which is closely linked to diabetic conditions [2, 3]. During liver fibrogenesis, RAGE can be upregulated when hepatic stellate cells are activated to transdifferentiate into myofibroblasts [4]. In the present study, hepatic VEGF expression was significantly increased along with elevated, although not significant, expression of RAGE in the LB-A, indicating a novel association of AGEs with hepatic gene expression that is closely related to liver fibrosis. Furthermore, hepatic VEGF gene transgenic rabbits showed many features similar to those observed in hemangiomatous disorders such as hemangioma-thrombocytopenia syndrome (Kasabach–Merritt syndrome) [7], implying that other vascular abnormalities can be caused by high AGE intake. Further studies are needed to examine whether VEGF expression is directly or indirectly affected by AGEs themselves and/or other components in the LB-A.

In non-diabetic subjects receiving an experimental diet containing AGEs, serum AGE levels reflected nearly 10% of the total AGEs in the diet, and returned to baseline levels 24 h after ingestion [8]. The present study showed no difference in serum AGE levels between LB-A and control groups, which was probably because blood samples were obtained 24 h after the final LB-A administration. In diabetic hyperglycemia, prolonged increase in glucose concentrations in retinal, renal and neural tissues facilitated AGE *de novo* synthesis in these tissues, resulting in diabetic complications [1]. In the present study, hyperglycemia was not observed, suggesting that hepatic AGE accumulation was not related to hyperglycemia but was due to exogenous AGEs internalized into liver tissues. Smedsrød et al. reported that approximately 80% of radiolabeled AGE-bovine serum albumin was recovered in several tissues after intravenous injection, and more than 90% of recovered radioactivity was detected in the liver 60 min after administration [17]. That can explain why AGEs in the LB-A accumulated predominantly in hepatic tissues, and affected VEGF expression in the liver rather than in the kidneys. The relationship between hepatic AGE accumulation and VEGF induction requires further investigation.

Probiotic *Lactobacillus* strains have been shown to have health-promoting potential, such as enhancing the host's immune response [14], improving intestinal barrier function [26], and reducing the risk factors of cancer [15] and diabetes [11, 25]. For such health benefits, various *Lactobacillus*-containing dietary products have been manufactured, and some have been designed as functional food items. With obvious advantages for convenience, *Lactobacillus* beverages are widely consumed by the Japanese population, and are even served as part of the hospital diet; however, our results suggest that these beverages facilitate hepatic VEGF expression and AGE accumulation, which

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in turn can outweigh their benefits in certain conditions.

In conclusion, a food item containing high levels of AGEs can serve to increase the risk of lifestyle-related diseases, particularly that of angiogenesis-associated diseases, through enhanced expression of VEGF in the liver. AGE content in foods and beverages should be taken into consideration for disease prevention, particularly in individuals at high risk of developing lifestyle-related diseases.

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