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Full paper

The specific localization of advanced glycation end-products (AGEs) in rat pancreatic islets

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ABSTRACT

Advanced glycation end-products (AGEs) are produced by non-enzymatic glycation between protein and reducing sugar such as glucose. Although glyceraldehyde-derived AGEs (Glycer-AGEs), one of the AGEs subspecies, have been reported to be involved in the pathogenesis of various age-relating diseases such as diabetes mellitus or arteriosclerosis, little is known about the pathological and physiological mechanism of AGEs *in vivo*. In present study, we produced 4 kinds of polyclonal antibodies against AGEs subspecies and investigated the localization of AGEs-modified proteins in rat peripheral tissues, making use of these antibodies. We found that Glycer-AGEs and methylglyoxal-derived AGEs (MGO-AGEs) were present in pancreatic islets of healthy rats, distinguished clearly into the pancreatic α and β cells, respectively. Although streptozotocin-induced diabetic rats suffered from remarkable impairment of pancreatic islets, the localization and deposit levels of the Glycer- and MGO-AGEs were not altered in the remaining α and β cells. Remarkably, the MGO-AGEs in pancreatic β cells were localized into the insulin-secretory granules. These results suggest that the cell-specific localization of AGEs-modified proteins are presence generally in healthy peripheral tissues, involved in physiological intracellular roles, such as a post-translational modulator contributing to the secretory and/or maturational functions of insulin.

1. Introduction

Diabetes mellitus (DM) induces many complications such as retinopathy, nephropathy and neuropathy.[1,2] These diseases have been suggested for involvement of advanced glycation end products (AGEs).[3–5]

AGEs are a heterogeneous, complex group of compounds that represent the ultimate product of multiple reactions occurring in several conditions, namely, in the chronic hyperglycemic state of DM. Non-enzymatic glycation begins with interaction and linked between the carbonyl group of a reducing sugar and an amino group of a protein.[3,6] The complex become to be early stage products such as a Schiff base and Amadori rearrangement products (e.g., HbA1c), and the compounds such as carboxymethyllysine (CML), carboxyethyllysine (CEL), and pentosidine (an AGE with autofluorescent properties) are formed through oxidation, dehydration and condensation.[3,6,7]

Recent studies have suggested that AGEs were produced not only from reducing sugars, but also from carbonyl compounds derived from the autoxidation of sugars and other metabolic pathways.[8,9] Five immunochemically distinct classes of AGEs (glucose-derived AGEs: Glc-AGEs, glyceraldehyde-derived AGEs: Glycer-AGEs, glycolaldehyde-derived AGEs; Glycol-AGEs, methylglyoxal-derived AGEs: MGO-AGEs, and glyoxal-derived AGEs: GO-AGEs) are present in sera of type 2 diabetic patients on hemodialysis.[10] And Glycer-AGEs are also called toxic AGEs (TAGE), which plays an important role in the

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pathogenesis of angiopathy in the diabetic patients.[4,11] These post-translational modifications of AGEs subspecies may accumulate by aging and be involved in the pathological processes in various diseases, such as DM,[3–5] Alzheimer's disease[12] and cancer.[13] However, the pathological consequence and physiological functions of AGEs accumulation are still poorly understood.

In this study, we produced 4 kinds of anti-AGEs subspecies-specific rabbit polyclonal antibodies to investigate the distribution of each AGE in healthy control rats as well as streptozotocin (STZ)-induced type 1 diabetic rats. The identification of AGEs existence in specific cells in detail must be the first step to understand the functional roles of them. Altogether, we demonstrate that AGEs are present in several peripheral tissues in normal rats. In particular, the Glycer-AGEs and MGO-AGEs were localized in α and β cells in the pancreatic islets, respectively. Therefore, we speculate that AGEs may have a specific role in the secretory processes in pancreatic islets.

2. Materials and methods

2.1. Experimental animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Okayama University, and performed in accordance with the guidelines of Okayama University on animal experiments. Adult male animals of Wistar rats and Japanese white rabbits at 12 weeks age obtained from SLC Japan (Tokyo, Japan). The experimental type 1 diabetes rats (STZ-induced diabetic rats) were prepared by intraperitoneal injection of 75 mg/kg streptozotocin (Wako, Osaka, Japan) dissolved in 0.05 M sodium citrate, pH 4.5. Healthy control rats were injected with sodium citrate. Forty-eight hours after streptozotocin injection, diabetic state was confirmed by the blood glucose levels higher than 300 mg/dL. Rats were sacrificed under anesthesia to obtain the peripheral tissues at 7 days after the streptozotocin injection. Rabbits were used for the productions of anti-AGEs antibodies (See below).

2.2. Synthesis of AGEs

AGEs-modified bovine serum albumin (BSA) (Sigma–Aldrich, St. Louis, MO, USA) was prepared as described previously.[14] BSA was incubated under sterile conditions with 0.2 M glyceraldehyde (Sigma–Aldrich), glycolaldehyde (Sigma–Aldrich), methylglyoxal (Sigma–Aldrich) or glyoxal (Tokyo Chemical Industry, Tokyo, Japan) in 0.2 M phosphate buffer, pH 7.4, at 37 °C for 7 days. Each of AGEs-BSA was dialyzed at 4 °C to remove free aldehyde.

2.3. Production of anti-AGEs-specific polyclonal antibody

Japanese white rabbits were immunized with AGEs-BSA emulsified with Freund's complete adjuvant (Wako). The booster injection with Freund's incomplete adjuvant (Wako) was administered 3 weeks later. After whole blood was collected from the immunized rabbits under anesthesia, the antisera titers were determined by immunoblot analysis as mentioned below. Immunoglobulin fractions were precipitated from the sera by ammonium sulfate as described previously.[15] After extensive dialysis, immunoglobulin was purified using MEP HyperCel (Pall, Port Washington, NY, USA). Anti-AGEs-specific antibodies were purified by using each of AGEs-BSA immobilized on Sepharose beads (GE Healthcare, Buckinghamshire, UK).

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as previously described.[16] ELISA plates were coated with each of 2 μg/mL AGEs and blocked with 1% skim milk. Each of rat anti-AGEs-specific polyclonal antibody was added to the well of ELISA plates. After overnight incubation at 4 °C, peroxidase-conjugated goat anti-rat IgG (Dako, Glostrup, Denmark) was added to each well. After 1 h incubation at room temperature, o-phenylenediamine (Wako) was added to each well. The colorimetric reaction was stopped with 3 M sulfuric acid. The absorbance was measured at 490 nm (Bio-Rad model 450 microplate reader; Bio-Rad, Hercules, CA, USA).

2.5. Immunohistochemistry

For immunohistochemical staining, paraffin-embedded peripheral tissues were prepared as previously described.[17] Each tissue was cut into paraffin sections at 5 μm thickness. After deparaffinization, the sections were boiled for the antigen activation at 120 °C for 10 min with 10 mM sodium citrate, pH 6.0. Subsequently, the sections were incubated with each of 2 μg/mL anti-AGEs-specific antibody (anti-Glycer-, anti-Glycol-, anti-MGO- and anti-GO-AGEs rabbit polyclonal antibodies), 3 μg/mL anti-insulin mouse monoclonal antibody (Cat. 2IP10 D3E7, HyTest, Turku, Finland) or 4 μg/mL anti-glucagon mouse monoclonal antibody (Cat. ab10988, Abcam, Cambridge, UK). To stain the vesicular structure, the sections were incubated with 10 μg/mL anti-VAMP2 (vesicle-associated membrane protein 2) mouse or rabbit monoclonal antibody (Cat. ab118899, Abcam or Cat. 13508, Cell Signaling, Danvers, MA, USA). The secondary antibodies used were an Alexa Fluor 488- or 555-conjugated goat anti-mouse or anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA, USA). The sections were also counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The sections were observed under fluorescent confocal microscopy (Biozero BZ8000; Keyence, Osaka, Japan).

2.6. Immunoblot analysis

Protein samples of AGEs-BSA were prepared for immunoblot analysis as previously described.[18,19] AGEs-BSA was denatured by boiling at 5 min with Laemmli-modified buffer [10% sodium dodecyl sulfate (SDS), 9.3% dithiothreitol (DTT), 30% glycerol, 0.35 M tris–Cl, pH 6.8]. Each of 1 μg AGEs-BSA sample was loaded onto 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto PVDF membranes. The membrane was blocked with 20% skim milk (wt/vol) and 1% normal goat serum, and subjected to incubation with 2 μg/mL rabbit polyclonal antibody against Glycer-AGEs, Glycol-AGEs, MGO-AGEs or GO-AGEs. Subsequently, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (MBL, Nagoya, Japan). The chemiluminescent reaction was started by the addition of Luminata Forte Western HRP Substrate (Millipore, Bedford, MA, USA), and the chemiluminescence was detected by ImageQuant LAS 4000 mini (GE Healthcare).

2.7. Statistical analysis

Statistical comparisons were performed using one-way ANOVA followed by the *post hoc* Bonferroni test with Ekuseru-Toukei 2010 (Social Survey Research Information, Tokyo, Japan). The mean values of data are shown along with the standard error. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Immuno-specificity of anti-AGEs polyclonal antibodies

Fig. 1A–D shows the specificity of each polyclonal antibody against AGEs-BSA adducts using immunoblot analysis. Mobility of

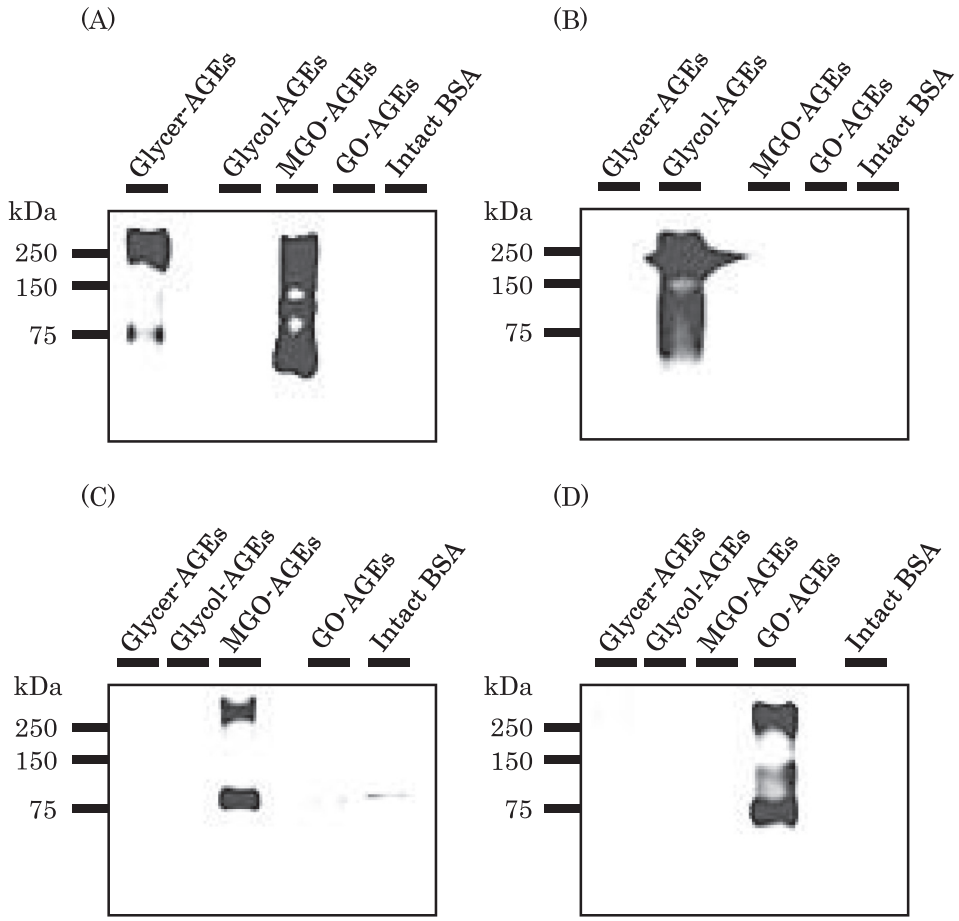

Fig. 1. Specificity and cross-reactivity of anti-AGEs antibody against each of AGEs-BSA by using immunoblot analysis. The indicated AGEs-modified BSAs were loaded onto polyacrylamide gel and subjected to immunoblot analysis with anti-Glycer-AGEs antibody (A), anti-Glycol-AGEs antibody (B), anti-MGO-AGEs antibody (C) or anti-GO-AGEs antibody (D).

AGEs showed ladder pattern, because AGEs may produce cross-linked structures between albumin molecules randomly. Anti-Glycer-AGEs antibody showed cross-reactivity for not only the Glycer-AGEs but also the MGO-AGEs (Fig. 1A), whereas other antibodies showed the specific reaction to each of the antigen (Fig. 1B–D).

Consistent with the results of immunoblot analysis in Fig. 1, ELISA also showed similar cross-reactivity of antibodies against each of the AGEs-BSA (Fig. 2).

3.2. Distribution pattern of AGEs in rat peripheral tissues

Positive immunostaining of Glycer-AGEs, which has the most toxic property among the AGEs subspecies arising in chronic hyperglycemia,[10] were observed in the brain, pancreas and stomach in the healthy control rats (Table 1). In peripheral tissues examined in this study, there seemed to be no difference in the AGEs distribution between the healthy control rats and the STZ-induced diabetic rats (Table 1).

To examine the distribution pattern of AGEs subspecies in rat pancreas, immunohistochemical staining for each of AGEs subspecies was performed (Fig. 3). We observed that Glycer-AGEs and MGO-AGEs were located in the pancreatic islets, but Glycol-AGEs and GO-AGEs were not (Fig. 3A). The staining pattern of anti-Glycer-AGEs antibody was clearly different from that of anti-MGO-AGEs antibody (Fig. 3A).

Glycer-AGEs were localized to the periphery of each pancreatic islet in the healthy control rats, which corresponded to the histological localization of α cells. In fact, double immunohistochemical staining for Glycer-AGEs and glucagon showed that both staining merged mostly (arrowheads in Fig. 3B). However, some glucagon-negative cells in the periphery of islets were positive for Glycer-AGEs (arrow in Fig. 3B). These Glycer-AGEs-positive cells are most likely the δ cells or PP cells judging from the histological feature. Double immunohistochemical staining for Glycer-AGEs and insulin did not show the co-localization of both antigens in the pancreatic islets (Fig. 3C).

On the other hand, immuno-reactivities of MGO-AGEs were merged completely with localization of insulin, indicating that these MGO-AGEs-positive cells are β cells histologically (Fig. 3D). We also observed that the co-localization of MGO-AGEs and insulin was located within the cytoplasmic granule-like structures in the β cells (higher magnification image of merged picture in Fig. 3D). Double immunohistochemical staining of MGO-AGEs and vesicle-associated membrane protein 2 (VAMP2), which is a marker protein of the vesicle membrane including the insulin-secretory granules,[20] indicated the co-localization of both antigens in the pancreatic islets, except for the islet periphery having the α cells (Fig. 3E). Co-localization of insulin and VAMP2 were also observed in the same areas of pancreatic islets (Fig. 3F). These results as a whole suggested the existence of MGO-AGEs-modified proteins in the insulin-secretory granules.

In pancreatic islets of the STZ-induced diabetic rats, the atrophic morphological defects were observed, accompanying marked decrease in the pancreatic β cells (Fig. 4B). These results are

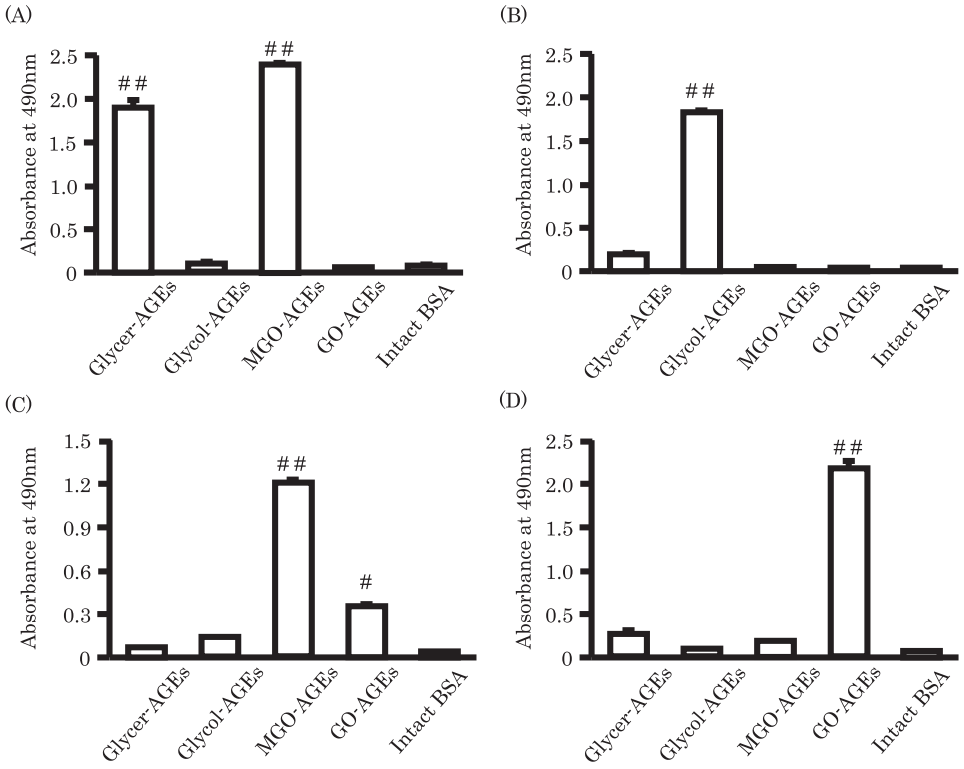

Fig. 2. Specificity and cross-reactivity of anti-AGEs antibody against each of AGEs-BSA by using ELISA. The indicated AGEs-modified BSAs were coated on ELISA plate and subjected to immunosorbent detection with anti-Glycer-AGEs antibody (A), anti-Glycol-AGEs antibody (B), anti-MGO-AGEs antibody (C) or anti-GO-AGEs antibody (D). Values represent the means ± SE, measured with triplicate wells for each group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the Intact BSA group.

Table 1
Distribution of Glycer-AGEs in rat peripheral tissues.

	Adipose	Brain	Colon	Duodenum	Eye	Heart	Kidney	Lung	Pancreas	Spleen	Stomach
Healthy	–	+	–	–	–	–	–	–	+	–	+
Diabetic	ND	+	ND	ND	ND	ND	–	–	+	ND	ND

The indicated tissues were prepared from the healthy control rats or the STZ-induced diabetic rats and subjected to immunofluorescent staining with anti-Glycer-AGEs antibody (Alexa Fluor 488). +: positive staining. –: negative staining. ND: not determined.

attributed to the β cell-specific cytotoxicity, since STZ is a toxic glucose analog which selectively accumulates in pancreatic β cells through the GLUT2 glucose transporter. However, the remaining α and β cells in the STZ-treated defective islets showed similar localization pattern and immuno-intensity of Glycer- and MGO-AGEs as compared with the healthy control rats, respectively (Figs. 3A and 4).

4. Discussion

AGEs are one of the post-translational modifications involved in various diseases, such as cancer,[13] Alzheimer's disease,[12] sarcopenia,[21,22] arthritis,[23] age-related macular degeneration (AMD),[24] non-alcoholic steatohepatitis (NASH)[25] and diabetes mellitus (DM).[3–5] Several antibodies against the specific AGEs subspecies have been utilized to investigate the role of AGEs modification. However, these antibodies have different cross-reactivity against each of AGE subspecies, because N-carboxymethyllysine (CML)-protein adduct is a major immunological epitope in AGEs-modified proteins.[26] In addition, the major source of CML is vir lipid peroxidation, not vir glycation.[27] Therefore, it is necessary for the future investigations to provide the more specific antibodies against the AGEs structures associated directly with the AGEs-attributed pathogenesis.

In this study, we produced 4 kinds of polyclonal antibodies against AGEs subspecies, glyceraldehyde-derived AGEs (Glycer-AGEs), glycolaldehyde-derived AGEs (Glycol-AGEs), methylglyoxal-derived AGEs (MGO-AGEs) and glyoxal-derived AGEs (GO-AGEs). Confirming the immuno-specificity of our antibodies by using the immunochemical and histological analysis, we made use of these antibodies to determine the localization of AGEs products in rat peripheral tissues, especially focused on the histological localization of Glycer-AGEs and MGO-AGEs in pancreatic islets.

The anti-Glycol-, MGO- and GO-AGEs antibodies showed clearly the antigen specificities by using ELISA and immunoblot analysis, whereas the anti-Glycer-AGEs antibody cross-reacted with both of Glycer-AGEs and MGO-AGEs (Figs. 1 and 2). This cross-reactivity may result from the immuno-affinity of the anti-Glycer-AGEs antibody against homologous structure between Glycer-AGEs and MGO-AGEs. However, in immunohistochemical staining of rat pancreas, the anti-Glycer-AGEs antibody showed the strong and specific sensitivity, which was clearly different from other 3 kinds of antibodies, the anti-Glycol-, MGO- and GO-AGEs antibodies (Fig. 3A). Taken together, these results indicate that our antibodies are available for immunohistochemical investigation.

AGEs are mainly generated under chronic hyperglycemic conditions such as diabetes mellitus (DM) or age-associated diseases such as Alzheimer's disease and atherosclerosis.[5,12,28–30] The

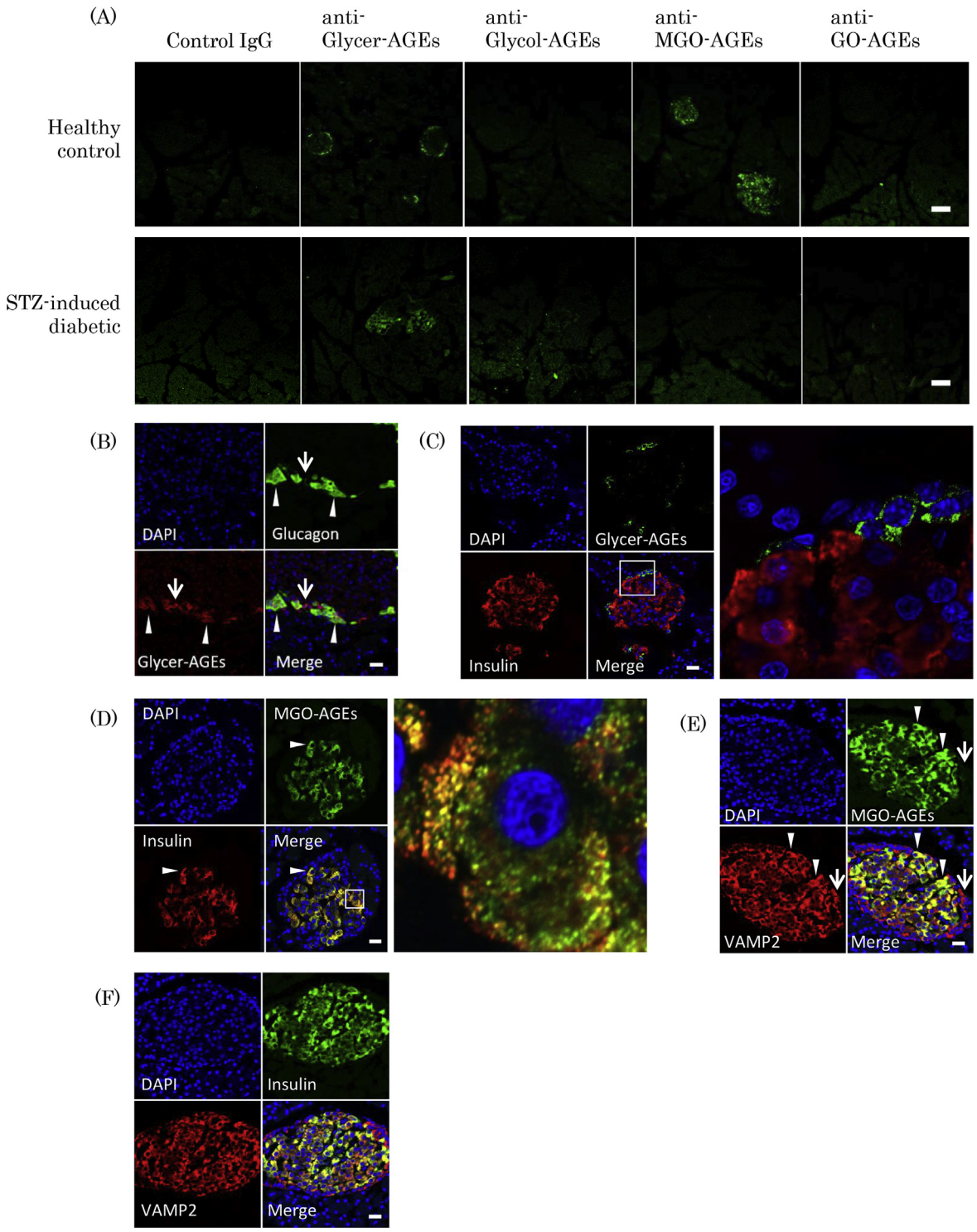

Fig. 3. Immunohistochemical localization of Glycer-AGEs and MGO-AGEs in rat pancreatic islets. Pancreatic sections were prepared from the healthy control rats or the STZ-induced diabetic rats and subjected to immunofluorescent staining for AGEs subspecies. Representative images for the localization of AGEs subspecies in the pancreatic islets are shown. (A) Control rabbit IgG and the indicated anti-AGEs antibodies against Glycer-AGEs, Glycol-AGEs, MGO-AGEs or GO-AGEs were used for the immunofluorescent staining (Alexa Fluor 488). The upper and lower panels show the pancreas of the healthy control rats and the STZ-induced diabetic rats, respectively. (B) Double immunohistochemical staining with anti-Glycer-AGEs antibody (Alexa Fluor 555) and anti-glucagon antibody (Alexa Fluor 488) is shown in the periphery of pancreatic islet derived from the healthy control rats. Arrowheads indicate the double-positive cells. Arrow indicates Glycer-AGEs-positive but glucagon-negative cell. (C) Double immunohistochemical staining with anti-Glycer-AGEs antibody (Alexa Fluor 488) and anti-insulin antibody (Alexa Fluor 555) is shown in the pancreatic islet derived from the healthy control rats. The right panel shows a magnified image of white square in the merged panel. (D) Double immunohistochemical staining with anti-MGO-AGEs antibody (Alexa Fluor 488) and anti-insulin antibody (Alexa Fluor 555) is shown in the pancreatic islet derived from the healthy control rats. The right panel shows a magnified image of white square in the merged panel. Arrowhead indicates the typical double-positive cell. (E) Double immunohistochemical staining with anti-MGO-AGEs antibody (Alexa Fluor 488) and anti-VAMP2 antibody (Alexa Fluor 555) is shown in the pancreatic islet derived from the healthy control rats. Arrowheads indicate the double-positive cells. Arrow indicates VAMP2-positive but MGO-AGEs-negative cell. (F) Double immunohistochemical staining with anti-insulin antibody (Alexa Fluor 488) and anti-VAMP2 antibody (Alexa Fluor 555) is shown in the pancreatic islet derived from the healthy control rats. The sections (B–F) were counterstained with DAPI. The scale bars indicate 100 μm (A) or 20 μm (B–F).

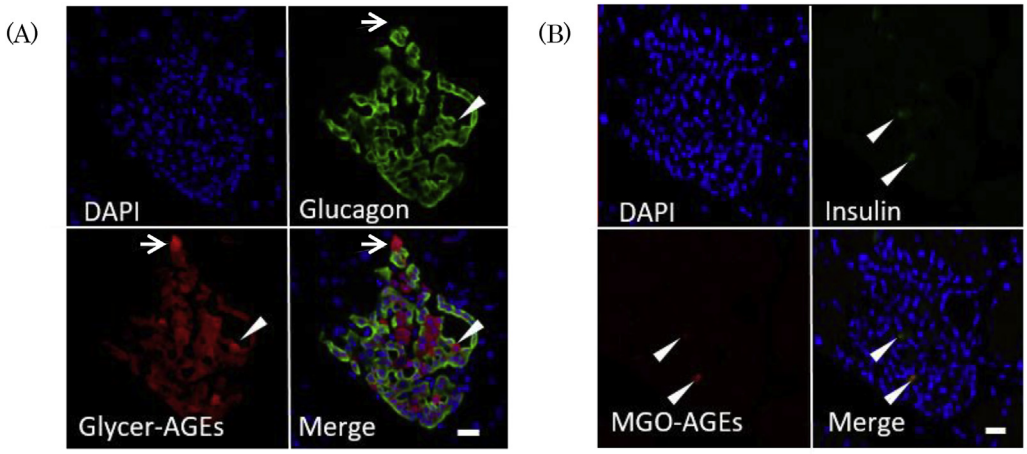

Fig. 4. Immunohistochemical localization of Glycer-AGEs and MGO-AGEs in the pancreatic islets of STZ-induced diabetic rats. Pancreatic sections were prepared from the STZ-induced diabetic rats and subjected to immunofluorescent staining for AGEs subspecies. Representative images for the localization of AGEs subspecies in the pancreatic islets are shown. (A) Double immunohistochemical staining with anti-Glycer-AGEs antibody (Alexa Fluor 555) and anti-glucagon antibody (Alexa Fluor 488) is shown in the atrophic islet derived from the STZ-induced diabetic rats. Arrowhead indicates the double-positive cell. Arrow indicates Glycer-AGEs-positive but glucagon-negative cell. (B) Double immunohistochemical staining with anti-MGO-AGEs antibody (Alexa Fluor 555) and anti-insulin antibody (Alexa Fluor 488) is shown in the atrophic islet derived from the STZ-induced diabetic rats. Arrowheads indicate the double-positive cells. The sections were counterstained with DAPI. The scale bars indicate 20 μm.

increased AGEs products in sera are considered as the major factor to induce various inflammatory complications due to vascular endothelial disorder.[6]

Takeuchi *et al.* have demonstrated that Glycer-AGEs have predominantly a toxic property among all AGEs subspecies,[4,10,11,31] which is termed toxic AGEs (TAGE). TAGE is involved in the pathogenesis of vascular complications in DM patients. Moreover, the ligand binding of TAGE through the receptor for AGEs (RAGE) alters intracellular functions, such as gene expression, release of pro-inflammatory molecules and production of reactive oxygen species (ROS) in various peripheral tissues, all of which may contribute to the pathological development observed in lifestyle-related diseases.

On the other hand, the findings in this study show that the Glycer-AGEs and MGO-AGEs were present in pancreatic islets of the healthy control rats, which were clearly distinguished into two types of islet cells, α cells and β cells (Fig. 3). Although the remarkable decrease in the number of insulin-secreting β cells and hyperglycemia were observed in STZ-induced rats, the localization and deposit levels of Glycer- and MGO-AGEs appeared not to be altered in the remaining α and β cells in the STZ-induced diabetic rats (Fig. 4).

These results suggest that the cell-specific localization of AGEs-modified proteins is related to healthy condition, involved in physiological intracellular functions. In Fig. 3D and E, the MGO-AGEs in pancreatic β cells were shown to be localized in the insulin-secretory granules. MGO-specific glycation may serve a critical role as post-translational modulation for the committed proteins in the insulin secretion or maturation.[32,33]

Previous reports have shown that Glycer-AGEs treatment to rat pancreatic β cells induces the cell injury by oxidative stress through the mitochondrial pathway,[34] and impairs glucose-induced insulin secretion.[35] These toxic effects are considered to result from the extracellular stimuli of Glycer-AGEs through the ligand binding for RAGE. As for an influence on cell function caused by intracellular AGEs-deposit, little is known about the molecular mechanism without the activation of RAGE signaling, at least in pancreatic islets.

Glycation contributes to an influence on surface charge on proteins leading to the structural and functional alterations, which in turn affects a protein–protein interaction and an affinity for hydrophilic structures.[36] Our present data suggests that the post-translational modifications by specific AGEs subspecies have a potential to influence on the physiological functions in the pancreatic α and β cells.

In this study, we have provided the valuable antibodies against specific AGEs subspecies for immunochemical detection *in vivo* and suggested the presence of AGEs-specific physiological processes in peripheral tissues. Further works are still required to understand the critical differences between pathogenic and physiological AGEs modifications in peripheral tissues through the lifespan.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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