Immunohistochemistry for Anti-diabetes Drug, Alogliptin Using a Newly Prepared Monoclonal Antibody: Its Precise Localization in Rat Small Intestine

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Knowledge of time sequence of localization of drugs in cells and tissues of animals may help in developing a better understanding of the actual overall pharmacokinetics of the drugs. We produced monoclonal antibody (mAb) against alogliptin (AG), a dipeptidyl peptidase-4 (DPP-4) inhibitor, conjugated to BSA with N-(γ-maleimidobutyryloxy)-succinimide. The mAb was specific for AG and did not cross-react with sitagliptin, vancomycin or amoxicillin. The mAb enabled us to develop an immunohistochemical method for detecting the localization of AG in the rat small intestine. One hour after a single oral administration of AG, immunohistochemistry revealed that the immunoreactivity of AG was observed in almost all of cells and tissues of the duodenum. The microvilli of the absorptive epithelial cells were moderately stained. The staining pattern of AG at jejunum and ilium was almost the same as that of duodenum, but the staining intensity, especially at absorptive epithelial cells and intestinal gland epithelial cells, became stronger towards the distal part of the small intestine. These results suggested that AG may be more actively absorbed from the lower part of the small intestine than in the upper part. It may affect the function of cells with membrane-bound DPP-4 because it was reported that membrane-bound form of DPP-4 exists in the microvilli of the absorptive epithelial cells.

Key words: alogliptin, immunohistochemistry, localization, intestine, rat

I. Introduction

Globally, the number of diabetic patients, which was 108 million in 1980, increased to 422 million in 2014 [35], ~4 times increase in 40 years. Diabetes is classified as type 1 diabetes when little or no insulin is produced and type 2 diabetes when insulin secretion and insulin action is insufficient. Majority of people are affected by type 2 diabetes [35]. Therapeutic agents for type 2 diabetes include sulfonylureas (stimulate insulin secretion from pancreatic β-cells), biguanides (reduce insulin resistance), α-glucosidase inhibitors, and incretin-related agents.

Recently, incretin-related agents such as dipeptidyl peptidase-4 (DPP-4) inhibitors and glucagon-like peptide (GLP)-1 receptor agonists are being widely used in the treatment of type 2 diabetes patients. The DPP-4 inhibitors augment the glucose-dependent insulin secretion through enhancement of the action of endogenous incretins, such as GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) by inhibiting DPP-4, a degrading enzyme of incretin [29]. Compared to the use of conventional drugs, such as sulfonylureas, the incretin-based therapies are considered to have a lower risk of hypoglycemia and weight gain, acute pancreatitis and pancreatic cancer [5, 6, 31]. However, there are reports that saxagliptin, a DPP-4 inhibitor, induced recurrent acute pancreatitis [23]. The DPP-4 inhibitors induced morphological abnormalities in the pancreas treated with incretin therapy [19]. Also there appears to be a statistical association between DPP-4 inhibitor use and
pancreatic carcinoma [27]. Although DPP-4 circulates in blood as a soluble enzyme [21, 24], the major fraction of the total body's DPP-4 is not localized in plasma, but is present in peripheral tissues in a membrane-bound form [15, 16, 18, 21]. Thus, knowledge of the time sequence of the localization of DPP-4 inhibitors in cells and tissues of animals would be useful in developing a better understanding of the mechanisms behind the action and/or adverse effects of the drugs and their appropriate usage. However, only a few reports about the cell and tissue localization of the DPP-4 inhibitors have been obtained by autoradiography using radiolabeled drugs [16, 20, 28].

For over 10 years, we have successfully developed immunohistochemical procedures for detecting cell and tissue localization of some drugs, such as daunomycin [11, 32], gentamicin [12], amoxicillin [13], and vancomycin [14]. We now report on the preparation and characterization of a specific monoclonal antibody to alogliptin (AG), one of the DPP-4 inhibitors, and the development of an IHC method for the localization of AG in the intestine of rats orally administered with the drug.

II. Materials and Methods

Preparation of immunogen (AG-GMBS-BSA conjugate)

The immunogen was prepared according to our previous method for anti-daunomycin serum using a heterobifunctional agent N-(γ-maleimidobutyryloxy)succinimide (GMBS; Dojindo Laboratories, Kumamoto, Japan) [9, 11]. Briefly, AG (2 mg, 5.9 μmol; Takeda Pharmaceutical Co. Ltd., Osaka, Japan) in 2.0 ml of 0.1 M phosphate buffer, pH 7.0; and 1.6 mg (5.7 μmol) GMBS in 0.5 ml tetrahydrofuran were mixed, constant stirred, and incubated at room temperature for 60 min, thus yielding a GMBS-acylated AG solution. The sample was centrifuged for 10 min at 2,000 rpm, and the supernatant was collected. Acetylmercaptosuccinyl BSA (AMS-BSA, 15 mg, approximately 0.1 μmol) was dissolved in 200 μl of 0.1 M phosphate buffer, pH 7.0, and incubated with 50 μl of 0.5 M hydroxylamine, pH 7.4, at room temperature for 10 min to remove the acetyl group. The resulting mercaptosuccinyl BSA (MS.BSA) was diluted with 1 ml of 0.1 M phosphate buffer, pH 7.0, and added immediately to GMBS-acylated AG supernatant and incubated for 60 min with slow stirring. The conjugate was applied to a 2.5 cm by 45 cm Sephadex G-75 column equilibrated with 10 mM phosphate buffer (pH 7.0) and eluted with the same buffer. The eluate, monitored at 280 nm, was collected in 3 ml fractions and the concentration of the conjugate was determined by Modified Lowery Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The peak fraction was used for immunization.

Preparation of anti-AG mAbs

Three five-week-old female BALB/c mice were injected intraperitoneally (i.p.) with 100 μg of AG-GMBS-BSA conjugate emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA). Subsequently, they received two injections of 50 μg of the conjugate emulsified in Freund's incomplete adjuvant at two-week intervals. Following immunization, antisera were collected, and antibody titers were evaluated with an enzyme-linked immunosorbent assay (ELISA) as described below. The mouse with the best immune response was selected for hybridization. It was given a fourth i.p. booster injection and sacrificed 4 days later.

Cell fusion and cloning

The spleen cells (2 × 10^6) from the immunized mouse and 3 × 10^7 myeloma cells (P3/NS-1) were fused with the help of polyethylene glycol, according to our previous method [11]. Cells suspended in HAT medium were dispensed into 96-well tissue culture plates (Corning, New York, NY, USA) at a density of 10^4 cells per well in which 10^5 feeder cells had been plated. From 10 to 20 days post-fusion, the wells were screened for reactivity using an ELISA method, as described below. Limiting dilutions of positive cultures were carried out two or three times to obtain monoclonality, and sub-isotyping of the mAbs was performed using a Mouse Monoclonal Sub-Isotyping Kit (American Qualex Manufactures, San Clemente, CA, USA).

ELISA method (Dilution ELISA)

ELISA was performed similarly to our previous method for anti-DM mAbs [11]. For screening clones producing antibody against AG-GMBS-BSA, the wells in microtiter plates were coated with 100 μl of the AG-GMBS-BSA conjugate (10 μg/ml) for 60 min at 37°C and then blocked the protein binding sites with 1% skimmed milk for 30 min at room temperature (RT). The wells were then incubated with serially diluted antisera or hybridoma culture supernatant for 90 min at 37°C, followed by goat anti-mouse IgG labeled with horseradish peroxidase (HRP) (whole IgG, diluted 1:1,000; Cappel, West Chester, PA, USA) for 60 min at 37°C. The amount of enzyme conjugate bound to each well was measured using 3,3',5,5'-tetramethylbenzidine (F. Hoffmann-La Roche Ltd., Basel, Switzerland) as a substrate, and the absorbance at 450 nm was read with an automatic ELISA analyzer (ImmunoMini NJ-2300; Nalje Nunc Int. Co. Ltd., Tokyo, Japan).

Inhibition ELISA

According to our previous method [11], wells in a microtiter plate were coated with the AG-GMBS-BSA conjugate and blocked with skimmed milk as described above. A fixed concentration of anti-AG monoclonal antibody AAG-78 mAb (1:50; 50 μl), and then 50 μl of AG-GMBS-BSA, vancomycin (VCM)-GMBS-BSA or amoxicillin (AMPC)-glutaraldehyde (GA)-BSA conjugates at various concentrations were added to the wells, and incubated for 90 min at 37°C, followed by incubation with HRP labeled
goat anti-mouse IgG (1:1,000) for 60 min at 37°C. The bound HRP activity was measured using the ELISA analyzer.

**Binding ELISA**

Compounds having primary amino groups such as AG, can be fixed to primary amino group of amino acids by GA. According to our previous method [14], wells in a microtiter plate coated with poly-L-lysine (30 μg/ml) were activated with 2.5% GA in 50 mM borate buffer, pH 10.0, for 60 min. The wells were subsequently incubated with test compounds at various concentrations for 60 min at RT. Excess aldehyde groups were blocked with 1% sodium borohydride. The wells were further incubated for 60 min with 1% skimmed milk, and then incubated overnight at 4°C with the primary antibody AAG-78, diluted 1:100 in phosphate-buffered saline (PBS; 10 mM phosphate buffer, pH 7.2, containing 0.05% Tween 80 (PBST). The wells were then incubated for 60 min at 37°C with HRP-labeled goat anti-mouse IgG (diluted 1:2,000). The bound enzyme activity was measured as described above.

**Animals**

Normal adult male Wistar rats (Kyudo Exp. Animals, Kumamoto, Japan), 200–250 g body weight, were used in this study. The principles of laboratory animal care and specific national laws were observed. The animals were housed in temperature- and light-controlled rooms (21 ± 1°C and 12 hr L:12 hr D) and had free access to standard food and tap water. Three rats were orally administered a single dose of 5 mg AG/kg of body weight. After 1 hr, the rats were anaesthetized with sodium pentobarbital (60 mg/kg; Abbott Laboratories, North Chicago, IL, USA) and perfused transcardially with PBS containing 5,000 IU of heparin sodium (AY Pharmaceuticals Co., Ltd. Tokyo, Japan) at 50 ml/min for 2 min at RT, and then with a freshly prepared solution of 2% GA in PBS for 6 min. The duodenum, jejunum and ilium were quickly excised and postfixed in the same fixative overnight at 4°C.

**Immunohistochemistry**

The IHC method was carried out essentially according to our previous methods [13, 14]. The postfixed specimens of the small intestine were subsequently embedded in paraffin in a routine way. The samples were cut into 5 μm-thick sections and then deparaffinized and rehydrated. The sections were consecutively treated with, 1) 6% hydrogen peroxide (Nacalai Tesque, Kyoto, Japan) in PBS for 30 min, 2) 2N HCl for 30 min, 3) 0.03 mg/ml protease (Type XXIV: Bacterial; Sigma-Aldrich Co. Inc., St. Louis, MO, USA) in PBS for different periods (15 min to 2 hr) at 30°C [7], 4) 5 mg/ml NaBH₄ (Sigma-Aldrich Co. Inc., St. Louis, MO, USA) in PBS for 10 min [3, 17]. After each process, the specimens were washed three times with PBS. Next, the specimens were blocked with a protein solution containing 10% normal goat serum, 1.0% BSA, and 0.1% saponin in TBS for 1 hr at RT and then directly incubated at 4°C overnight with AAG-78 mAb, diluted 1:20 in TBS supplemented with 0.1% Triton X-100 (TBST). The sections were washed with TBST three times, 5 min at a time, and then incubated with Simple Stain Rat MAX-PO (M) (Nichirei Bioscience Inc., Tokyo, Japan) for 2 hr at RT. After rinsing with TBS, the site of the antigen-antibody reaction was revealed by using the substrate 3,3'-diaminobenzidine, tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan) and H₂O₂ for 10 min.

Two types of negative control experiments, conventional control experiment and absorption control experiment, were performed. In conventional control, the sections were exposed to normal mouse IgG diluted to 0.5 μg/ml with TBST instead of the primary mAb. In absorption control, the diluted primary mAb was preabsorbed with 30 μg/ml AG-GMBS-BSA, more than 100 fold in molar ratio as compared to antibody, before reacting with the sections.

**III. Results**

**Generation and specificity of mAb to AG**

From the fusion experiment, 150 hybridoma lines were produced. Two hybridoma lines (clone no. 73, 78) secreted antibodies that bound to the AG-GMBS-BSA conjugate but did not recognize BSA when screened by ELISA supplemented with BSA (1 mg/ml), which left anti-BSA antibodies out. Subclones of the hybridoma obtained by limiting dilution of clones 73 and 78 were found to produce an antibody. They secreted antibodies into the culture supernatant. We use hybridoma culture supernatants as mAbs and called them AAG-73 and AAG-78, respectively. Sub-isotype of AAG-73 and 78, determined by a Mouse Monoclonal Sub-Isotyping Kit (American Qualex Manufactures, San Clemente, CA, USA), was IgG1 for both. Since the results of ELISA and immunohistochemistry with these two mAbs were almost the same, only the results of AAG-78 were described below. The IgG concentration of AAG-78 was 9.23 μg/ml as determined by mouse IgG immunoassay kit (PerkinElmer Inc., Waltham, MA, USA).

**Dilution ELISA**

Microtiter wells coated with AG-GMBS-BSA conjugate (10 μg/ml) were used to test for antibody binding using serial dilutions of AAG-78 mAb (hybridoma culture supernatants). As shown in Figure 1, significant binding activities were observed with AAG-78 mAb in dilutions of less than 1: 500 (Fig. 1). No antibody binding was seen with type-matched (IgG1) mAb for polyamines (ASPM-29), known to be specific to spermine and spermidine [10] (data not shown).

**Inhibition ELISA**

Inhibition ELISA was achieved by the principle of competition between AG conjugates (AG-GMBS-BSA)
(free in solution), and a fixed amount of AG-GMBS-BSA coated on ELISA plates for the limited number of binding sites on the AAG-78 mAb. Calibration curves were plotted showing the relationship between the concentrations of the analytes and the percentage of bound mAb, giving dose-dependent inhibition curves with the conjugate AG-GMBS-BSA in the range between 0.1 nM and 100 nM (Fig. 2). The dose required for 50% inhibition of binding was used as an indication of the strength of inhibition. This dose (IC\textsubscript{50}) was 0.4 nM with AG-GMBS-BSA. Almost no inhibition occurred with VCM-GMBS-BSA, and AMPC-GA-BSA tested even at concentrations of less than 1 μM (Fig. 2).

**Binding ELISA**

The binding ELISA simulates the IHC of tissue sections on the basis of the principle of coupling of the amino group of the analytes to the wells of a microtiter plate activated with poly-L-lysine and GA and incubation of the wells by the indirect immunoperoxidase method. As shown in Figure 3, analysis of the relationship between the concentration of each of the analytes applied to the wells and the bound HRP activity produced a dose-dependent curve, with AG concentrations ranging from 0.3 μM to 10 μM. Almost no immunoreaction with sitagliptin (SG), VCM or AMPC was observed (Fig. 3).

**Localization of AG in rat small intestine**

Immunohistochemistry for drugs requires GA fixation. Therefore, there is a difference in the intensity and localization of signal obtained by intensity of protease digestion to increase the permeability of the antibody. Though, increasing the protease digestion generally increases the signal intensity and appears new positive sites, the signal observed in no or weak digested sections occasionally attenuates or disappears [13]. In this study, a strong signal
was obtained in 60 min of protease digested sections and the signal observed in no or weak digested specimens did not disappear. Therefore, we show only the results of 60 min protease treatment below.

The duodenum

In the duodenum specimens, IHC revealed faint to somewhat strong staining for AG in almost all the cells and tissues of the sections (Fig. 4b). The brush borders of the absorptive epithelial cells and intestinal glands were weakly stained (Fig. 4b, c). The lamina propria in the villi stained somewhat strong but that around the intestinal glands and submucosa were hardly stained (Fig. 4a, b). The smooth muscles were moderately stained (Fig. 4b). A small number of AG positive cells were found in the intestinal gland epithelium (Fig. 4h).

The jejunum

Absorptive epithelial cells and lamina propria in villi showed stronger immunoreactivity than those of duodenum (Fig. 4d). In particular, the staining intensity of the microvilli of absorptive epithelial cells had increased significantly compared to that of the duodenum (Fig. 4c, e). However, the immunoreactivity of the intestinal gland, lamina propria around glands, muscularis mucosae, submucosa and muscle layer was almost similar to that of the duodenum (Fig. 4d). The AG positive cells in the intestinal gland epithelium were also observed in the jejunum (Fig. 4i). The nerve cells of Meissner’s plexus (in the submucosa) and Auerbach’s plexus (between muscle layers) also showed positive signals (Fig. 4j–m).

The ileum

The sections showed moderate to very strong staining
for AG in almost all the cells and tissues (Fig. 4f). The absorptive epithelial cells stained more strongly than not only microvilli but also cytoplasm compared to duodenum and jejunum (Fig. 4c, e, g). The intestinal gland epithelial cells and luminal contents also showed positive (Fig. 4n).

The negative control experiments

The conventional IHC staining controls were all negative (Fig. 4o). The absorption controls showed that addition of AG-GMBS-BSA at a concentration of 30 μg/ml into the primary antibody solution abolished all staining (Fig. 4p).

IV. Discussion

Dipeptidyl peptidase-4 is a serine protease also known as the surface antigen CD26 of lymphocytes. It has been reported that in addition to lymphocytes, this enzyme shows high activity in the small intestine, kidney, liver, pancreas, and vascular endothelial cells [15, 18, 21]. It also circulates as a soluble form in the plasma [21, 24]. Dipeptidyl peptidase-4 rapidly inactivates the incretins that promote the secretion of insulin from islet β cells in a blood glucose-dependent manner and is closely involved in the onset of type 2 diabetes. Therefore, inhibition of DPP-4 activity suppresses the degradation of incretins and increases their effective concentration, which is considered to be effective as a therapeutic strategy for type 2 diabetes. New classes of antihyperglycemic agents are being used as DPP-4 inhibitors, and a number of drugs have been developed and used clinically [4, 26].

Alogliptin benzoate was approved for clinical use in the treatment of type 2 diabetes in Japan in April 2010 with the trade name Nesina [29]. Alogliptin is rapidly absorbed in the small intestine [22, 34]. Consistent with the rapid absorption of AG, inhibition of plasma DPP-4 activity occurred rapidly (15 min post-dose) after the administration of a single oral dose [22]. Alogliptin undergoes limited metabolism, and enzymes CYP2D6 and CYP3A4 are involved. The active metabolite M-I (N-demethylated) accounts for less than 2% of AG concentration in the urine, while the inactive metabolite M-II (N-acetylated) accounts for less than 6% [2, 30, 34]. Most of AG is excreted unchanged in the urine (60% to 71%) [34].

In this study, we prepared anti-AG mAb, characterized its specificity and developed an IHC procedure for determining the sites of AG accumulation in the rat small intestine, which is the main organ responsible for drug absorption. The AAG-78 mAb was demonstrated to be specific to AG by the inhibition and binding ELISA. Inhibition ELISA showed that antibody binding was strongly inhibited with AG-GMBS-BSA, but not at all with VCM-GMBS-BSA or AMPC-GA-BSA. The results of binding ELISA showed that the mAb binds to AG-GA-lysine but could not bind to SG, VCM and AMPC-GA-lysine. These results strongly suggest that AAG-78 mAb can recognize the drug very specifically and could be useful for IHC studies of drug localization in GA-fixed tissues.

The staining pattern of AG at each site of the small intestine was almost the same, and positive for absorptive epithelial cells, intestinal gland epithelial cells, connective tissue, nerve cells, and smooth muscle tissue. The staining intensity became stronger towards the distal part of the small intestine. The signals seen in the cytoplasm of absorptive epithelial cells and connective tissues of lamina propria mucosa suggest that AG is taken up into the body via epithelial cells, although its mechanism is unknown. Since the staining intensity of these sites becomes stronger towards the lower part of the small intestine, it is considered that it is more actively absorbed from the lower part than in the upper part of the small intestine. Further, there are reports that the membrane-bound form of DPP-4 exists in the microvilli of absorptive epithelial cells, and they are expressed more in the jejunum and ileum than in the duodenum [1, 25]. Therefore, it is highly probable that the strongly positive response seen at the brush border of absorptive epithelial cells represents both absorbed AG and AG binding to DPP-4. Since the DPP-4 in microvilli membrane is involved in degradation and uptake of peptides by epithelial cells, AG may affect the absorption of proteins and peptides from the small intestine.

The intestinal gland epithelial cells and luminal contents of the gland also showed positive. It is unlikely that the drug will be taken from the lumen side of the cells against the flow of intestinal fluids. Therefore, it is highly probable that the drug in the cells is absorbed from the basal side of the glandular epithelial cells, which is taken up from the absorptive epithelial cells and carried into the connective tissue and the drug in the lumen is secreted from glandular epithelial cells. There may possibly be a local drug circulation system through which it is secreted into the lumen of the small intestine with the intestinal fluids, and it is also taken up from the absorptive epithelial cells.

There are various endocrine cells called basal-granulated cells in the gastrointestinal tract epithelium [8, 33]. They secrete diverse hormones which affect the secretion of gastrointestinal fluid, pancreatic juice or bile, bowel motility and glycemic control. Alogliptin positive cells scattered in the intestinal gland epithelium are considered to be basal-granulated cells, because they showed characteristic pyramidal or spindle-like morphology. In addition, AG was also localized in nerve cells of Meissner’s plexus and Auerbach’s plexus. It is known that transporters are involved in drug uptake into these cells, but the detailed mechanism is unknown. These results suggest that the drug may affect intestinal secretion and motility, and may cause adverse effects such as constipation and ileus.

In conclusion, our study clearly demonstrates that the anti-AG mAb produced against GMBS-conjugated AG was very specific for AG and IHC using this mAb was very useful for analyzing the localization of AG in cells and tissues. This method revealed that the sites of AG accumulation closely correlate with the sites where the membrane-
bound form of DPP-4 occurs in the cells of the rat intestine. Thus, it was suggested that AG may affect the function of various cells with membrane-bound DPP-4.

V. Conflicts of Interest
The authors declare no conflicts of interest.

VI. Abbreviations
AG, alogliptin; DPP-4, dipeptidyl peptidase-4; GLP, glucagon-like peptide; GIP, glucose-dependent insulino-tropic polypeptide; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; GMBS, N-(γ-maleimidobutyryl)oxysuccinimide; GA, glutaraldehyde; TBST, TBS supplemented with 0.1% Triton X-100

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