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Evaluation of immunohistochemical staining for glucagon in human pancreatic tissue

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Immunohistochemistry (IHC) and immunofluorescence (IF) staining techniques are important diagnostic tools of anatomic pathology in the clinical setting and widely used analytical tools in research laboratories. In diabetes research, they are routinely used for the assessment of beta- and alpha-cell mass, for assessment of endocrine cell distribution within the pancreas, for evaluation of islet composition and islet morphology. Here, we present the evaluation of IHC techniques for the detection of alpha-cells in human pancreatic tissue. We compared the Horse Radish Peroxidase (HRP)-based method utilizing DAB Peroxidase Substrate to the Alkaline Phosphatase (AP)-based method utilizing Vector Red substrate. We conclude that HRP-DAB staining is a robust and reliable method for detection of alpha-cells using either rabbit polyclonal or mouse monoclonal anti-glucagon antibodies. However, AP-Vector Red staining should be used with caution, because it is affected by the dehydration with ethanol and toluene preceding the mounting of slides with Permount mounting medium. When AP-Vector Red is a preferable method for alpha-cell labeling, slides should be mounted using aqueous mounting medium or, alternatively, they could be air-dried before permanent mounting

Keywords: Diabetes, Glucagon, Human pancreas, Immunofluorescence, Immunohistochemistry

Introduction

Immunohistochemistry (IHC) and immunofluorescence (IF) are standard diagnostic tools used widely in clinical and research laboratory settings. Pathological diagnosis of tissue sections is dependent upon reliable and reproducible staining techniques. The quality of staining is even more critical when quantitative staining analysis of tissue sections is performed.

Historically, staining of only one hormone per tissue section was performed, with DAB peroxidase staining being commonly employed and proven to be robust and reliable. However, more recently, double hormone staining has become more commonly utilized and offers a number of advantages. First, fewer tissue sections are required, which is a particular advantage when limited availability of human tissue is a factor. Second, it is useful to be able to visualize the distribution of two cell types side by side on the same section. However, adding a second hormone requires the use of a second color development system that is distinct and a clear contrast from DAB peroxidase. For this reason, AP-Vector Red is a popular choice.

However, we observed that, when using the Alkaline Phosphatase (AP) based method utilizing Vector Red

substrate for staining of alpha-cells in human pancreatic sections, some of the color was lost. We set out to determine at what stage and under what conditions this color loss occurred.

Materials and Methods

Tissue

Human pancreas specimen 6004 was procured from a brain-dead organ donor by the Juvenile Diabetes Research Foundation (JDRF) Network for Pancreatic Organ Donors with Diabetes (nPOD) coordinated through the University of Florida in Gainesville, FL, USA. All procedures were in accordance with federal guidelines for organ donation and the University of Florida Institutional Review Board. A surgically removed human tissue specimen was obtained in accordance with the University of California at Los Angeles Institutional Review Board approval, fixed in 4% phosphate buffered paraformaldehyde overnight at +4°C, and embedded in paraffin. Four millimeter sections were used for staining.

Antibodies

The following primary antibodies were used: Mouse monoclonal anti-glucagon clone K79bB10 from Sigma (St. Louis, MO, USA; Cat# G2654; Lot# 090M4826); rabbit polyclonal anti-glucagon serum from Immunostar (Hudson, WI, USA; Cat #20076; Lot#136027); guinea

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pig anti-insulin from Abcam (Cambridge, MA, USA; Cat#ab7842). Anti-glucagon antibodies were diluted 1:2000. Anti-insulin antibody was used at 1:100 dilution.

Detection antibodies were from Jackson ImmunoResearch (West Grove, PA, USA): Biotin-SP-F(ab')₂ Fragment Donkey Anti-Rabbit IgG (H+L) (Cat#711-066-152); Biotin-SP-F(ab')₂ Fragment Donkey Anti-Mouse IgG (H+L) (Cat#715-066-150); Cy3-F(ab')₂ Fragment Donkey Anti-Rabbit IgG (H+L) (Cat#711-166-152); FITC-F(ab')₂ Fragment Donkey Anti-Guinea pig IgG (H+L) (Cat#706-096-148). Detection antibodies were reconstituted in water with glycerol according to the manufacturer instructions, stored at -20°C, and used diluted at 1:100.

For dot blot, we used HRP-goat anti-rabbit antibody from Life Technologies (Carlsbad, CA, USA; Cat#65-6120) diluted at 1:3000.

Staining kits and other reagents

Reagents for buffers, toluene for tissue defatting and dehydration, bovine serum albumin (BSA) (Cat#A7030), and Harris Hematoxylin (Cat#HHS32) were from Sigma (St. Louis, MO, USA). From Vector Laboratories (Burlingame, CA, USA) there were: Antigen Unmasking Solution, Citric Acid Based, pH 6.0 (Cat#H-3300); Hematoxylin QS (Cat#3404); ABC-VECTASTAIN Standard Kit (Cat#PK-4000) and DAB Peroxidase Substrate Kit, 3,3'-diaminobenzidine (Cat#SK-4100); VECTASTAIN-ABC-AP Standard Kit (Cat# AK-5000), and Vector Red AP Substrate (Cat#SK-5100); Vectashield with DAPI (Cat#H-1200). Permout mounting medium was from Fisher (Waltham, MA, USA; Cat# SP15-100).

Peptides

Glucagon synthetic peptide was purchased from Sigma (St. Louis, MO, USA; Cat#G2044). GLP-1 (1-37) and GLP-1(7-36 Amide) were from Bachem (Torrance, CA, USA; Cat#H-5552 and Cat#H-6795, respectively). Glucagon was reconstituted in 0.05M Acetic Acid and GLP-1 peptides were reconstituted in water at 1mg/ml. Aliquots were stored at -20°C until further use.

Dot blot

Stock solutions of peptides were diluted in water and blotted at 0.2 µg/2 µl/spot on nitrocellulose membrane (Protran from Whatman, Maidstone, UK). Membranes were allowed to air-dry, then blocked in 5% milk (BioRad, Hercules, CA, USA) in TBS-T (TBS, 0.1% Tween-20) for 1 hour at room temperature, incubated overnight at +4°C with the primary antibody diluted in 5% BSA in TBS-T, washed, incubated with the detection antibody diluted in 5% milk in TBS-T for 1 hour at room temperature. Proteins

were visualized by enhanced chemiluminescence (BioRad, Hercules, CA, USA; Cat#170-5040) using the Labworks software (Waltham, MA, USA; UVP).

Antibody blocking with peptide

Antibody was diluted in TBS-T, and incubated at room temperature in the absence or presence of the peptide. Diluted rabbit anti-glucagon serum (1:200) was incubated with 10mg/ml of peptide for 6 hours at room temperature. After incubation with the peptide, the mixture was spun for 10min at 15 000g and diluted to 1:2000 before application to the sections or to the dot blot membrane. In other experiments, diluted rabbit anti-glucagon serum (1:2000) was incubated with 100mg/ml of glucagon peptide or GLP-1 7-36 amide for 12 hours at room temperature, spun and used for dot blot and staining.

Staining procedure

Slides were defatted in two changes of toluene (10 minutes each) followed by 10 minutes incubation in 100% ethanol, then 5 minutes in each 95% ethanol, 70% ethanol and water. Sections were submitted to heat-induced antigen retrieval in citrate buffer at pH 6.0, using microwave: three cycles for 5 minutes at power level 5 followed by a 30 second break, cooled to room temperature for 1 hour, then soaked in Soaking Buffer (TBS, 0.4% TX100) for 30 minutes on ice, and washed once with TBS.

For IHC, slides then were treated for 30 minutes at room temperature with 10% methanol and 3% H₂O₂ in TBS, blocked with BSA (Tris B: 0.1M Tris, 0.85% NaCl, 0.1% Triton X-100, 2% BSA, pH 7.4-7.6), and incubated, with the primary antibody diluted in Tris B buffer overnight at +4°C. After washing with TBS-T, slides were incubated, with the detecting antibody diluted in Tris B buffer for 1 hour at room temperature, and staining was completed using ABC kits and enzyme substrates from Vector according to the manufacturer instructions. After the color development, sections were counterstained with hematoxylin and images of islets and ducts were taken from the different areas of the section. The same areas were imaged after slides were dehydrated by incubation for 3min in each 70, 95, and 100% ethanol followed by 5min in two changes of toluene, and mounted using Permout.

For IF staining, after antigen retrieval slides were incubated in Soaking Buffer for 30 minutes, blocked using Blocking Buffer (TBS, 3% BSA, 0.2% Triton) for 1 hour, then incubated with the primary anti-glucagon antibody diluted in Antibody Buffer (TBS, 3% BSA, 0.2% Tween-20) overnight at +4°C. After washing in TBS-T, slides were incubated with the detecting antibody diluted in Antibody Buffer for 1 hour at room temperature, and then with anti-insulin antibody overnight at +4°C, followed

by the detecting antibody for 1 hour at room temperature. Slides were mounted using Vectashield with DAPI.

Imaging

Immunohistochemistry-stained slides were imaged at 10× (islets) and 20× (ductal areas) using an Olympus Magnafire SP Camera attached to an Olympus CX41 Microscope (Center Valley, PA, USA). Immunofluorescence-stained slides were imaged at 20× magnification using a Leica fluorescent microscope DM6000 (Buffalo Grove, IL, USA) and the OpenLab software (Perkin Elmer, Waltham, MA, USA).

Results

Alpha-cell function and mass in health and disease have been a focus of intensive research in recent years. In this brief technical report, we have evaluated the approaches used for immunohistochemical staining of alpha-cells in human pancreas. The ABC-VECTASTAIN Standard Kit and DAB Peroxidase Substrate Kit are commonly used for insulin staining of human and rodent pancreatic sections to measure fractional beta-cell area and beta-cell mass. When double staining is required for the assessment of beta-cell proliferation or apoptosis, we have used the AP-Vector Red for insulin staining in combination with HRP-DAB staining for nuclear markers. According to the manufacturer, sections stained with the Vector Red substrate can be dehydrated, cleared, and permanently mounted. In our hands, AP-Vector Red insulin staining is consistent and reliable. The attempt to use this approach for staining of alpha-cells however was not satisfactory.

The comparison of glucagon staining in adjacent sections of human pancreas revealed significant differences in the pattern of glucagon staining by IF and by IHC using VECTASTAIN-ABC-AP Standard Kit and Vector Red AP Substrate (Fig. 1). In the AP-Vector Red stained section, the number of glucagon positive cells per islet was much lower (Fig. 1D vs A), and glucagon staining of single cells and cell clusters in the ductal areas was not detected (Fig. 1E vs B and F vs C).

Before starting to investigate the reason for such differences in IF versus AP-Vector Red alpha-cell staining techniques, we tested the specificity of the rabbit anti-glucagon serum. We pre-incubated serum with either synthetic glucagon peptide or most closely related control peptides, and then performed a binding test using dot blot. Dot blot results presented in Fig. 2A demonstrate that antibody interacted with synthetic glucagon peptide, but not the related controls (synthetic GLP-1 (1-37) and GLP-1 (7-36 Amide)). Moreover, pre-incubation of serum with glucagon peptide, but not control peptides, prevented binding to blotted glucagon peptide (Fig. 2A) and abolished immunostaining of tissue (Fig. 2B).

After antibody specificity was confirmed, we set out to establish at what step of the IHC protocol glucagon staining was lost. We monitored the same islets and ductal areas at the following points of the staining procedure: (1) immediately after color development; (2) after the hematoxylin counterstain; (3) after dehydration, and (4) after mounting with Permount. We found that the Vector Red was washed out during the dehydration step before cover slipping (Fig. 3, Panel I: D vs A, E vs B, F vs C). The loss of color in islets was not always obvious and in some cases could have been revealed only by the side-by-side comparison of the images of the same islets in sequential sections. Glucagon staining in the ductal areas was completely gone after dehydration and cover slipping. The disappearance of staining could have been easily missed because glucagon-positive cells in ductal areas are relatively rare.

The modifications of the staining protocol such as (1) changing Harris Hematoxylin routinely used in the lab to the Hematoxylin QS as recommended by Vector; (2) shortening of dehydration steps; (3) skipping 70% alcohol step; or (4) usage of Xylene instead of Toluene as recommended by the manufacturer did not result in color preservation in islets or ducts. However, the color loss was prevented if slides were air-dried after the color development, and then permanently mounted (not shown).

To determine whether use of a different glucagon antibody would eliminate the problem, we performed staining using mouse monoclonal anti-glucagon antibody instead of rabbit serum. We again monitored and imaged islets and ductal areas before and after dehydration and mounting with Permount. As illustrated in Fig. 4, after the dehydration and mounting, staining was again somewhat weaker in the islets (Fig. 4, Panel I: D vs A), and completely lost in ducts (Fig. 4, Panel I: E vs B and F vs C).

The loss of glucagon staining was observed in surgical tissue procured at UCLA (Fig. 1) and tissue harvested from the nPOD brain dead organ donor (Figs. 3 and 4). Diabetic status did not seem to influence the loss of staining, as the surgical tissue was from a subject with T2D (Fig. 1) and nPOD procured tissue was from a non-diabetic donor (Figs. 3 and 4). Similar loss of glucagon staining was detected also in AP-VectorRed stained pancreatic sections containing glucagon expressing endocrine tumor (Fig. 5).

For comparison, HRP-DAB glucagon staining was performed on sections adjacent to ones presented in Panel II of Figs. 3–5, and the same islets and ductal areas were monitored and imaged. In contrast to AP-VectorRed staining, the HRP-DAB staining using either mouse or rabbit anti-glucagon antibody was not affected by the dehydration process in all tissues stained (Compare Panel II to Panel I in Figs. 3–5).

In summary, ABC-VECTASTAIN Standard Kit and DAB Peroxidase Substrate Kit is a reliable approach for

Figure 1 Glucagon staining pattern in adjacent sections of surgical human pancreatic tissue stained by immunofluorescence (IF) (A–C, Glucagon, red; nuclei, blue DAPI) and by immunohistochemistry (IHC) (D–F; VECTASTAIN-ABC-Alkaline Phosphatase (AP) Standard Kit and Vector Red AP Substrate) using rabbit anti-glucagon serum. Circles indicate the area where the staining was significantly weaker, and arrows point to the areas where the stain was absent in the section stained by IHC. Scale bars: (A, C, D and F) 50 μ m; (B and E) 100 μ m.

glucagon staining in human pancreas tissue sections. In contrast, glucagon staining utilizing AP-Vector Red is sensitive to the dehydration with ethanol and toluene preceding the mounting of slides with Permount mounting medium. When AP-Vector Red is a preferable method for alpha-cell labeling, slides should be mounted using aqueous mounting medium or, alternatively, they could be air-dried before permanent mounting.

Discussion

Glucagon-secreting alpha-cells and insulin-secreting beta-cells make up the majority of the endocrine cell population within the pancreatic islets, and they work together to maintain glucose homeostasis. Differences in distribution of alpha- and beta-cells between human islets and the commonly employed rodent models in health have been well documented.^{1–3} In humans with type 2 diabetes, however, these cellular proportions are disrupted due to a loss of beta-cells and deposition of islet amyloid within

the pancreatic islets.⁴ There has been uncertainty about whether the alpha-cell population undergoes changes in type 1 and type 2 diabetes. Some groups report an increase in alpha-cell mass in type 2 diabetes,^{5–8} and one of those same groups, in a much larger study, later reported no change.⁹ Several groups report no change in alpha-cells in type 1 diabetes.^{5,10}

In recent years, there has been increasing interest in the alpha-cell population, the regulation of glucagon secretion and the precise role of glucagon in glucose homeostasis.¹¹ The development and relationship between the alpha- and beta-cell mass in humans is also an area of active research.¹² In addition, a novel compartment within the pancreatic ductal tree, the pancreatic duct glands (PDGs), has been recently recognized and characterized.¹³ The PDGs have been suggested as harboring a pancreatic stem cell niche, and this compartment is therefore regarded as an exciting focus in the search for new therapeutic approaches for the treatment of diabetes.

Figure 2 Confirmation of specificity of rabbit polyclonal anti-glucagon antibody by dot blot (A) and immunostaining (B). The antibody interacts with glucagon peptide, but not control peptides that could be generated from the same gene product (A). The glucagon peptide, but not control peptides, blocks immunostaining (B) and interaction with the blotted glucagon peptide (A). Immunostaining was performed in the serial sections from the same block of tissue (nPOD 6004-07) by immunofluorescence (IF) (glucagon, red; insulin, green; nuclei, blue DAPI). To block binding, diluted anti-glucagon serum was pre-incubated with 10mg/ml (A) or 100mg/ml (B) of peptides (see Materials and Method sections for details). Scale bar 50µm all panels.

In order to study changes in alpha- and beta-cell mass in development and disease, it is necessary to have reliable and reproducible staining methods. Immunofluorescent staining is a sensitive method for detecting signal presence, although there are definite drawbacks when large tissue sections need to be scanned and analyzed. Fluorescent

tiling can be problematic with bleaching of the sample, and uneven background staining presents issues for analysis. Therefore, it is often more convenient to use IHC and analyze sections using bright field microscopy and scanning.

Figure 3 Images of the adjacent pancreatic sections stained with the rabbit anti-glucagon serum using either VECTASTAIN-ABC-Alkaline Phosphatase (AP) Standard Kit and Vector Red AP Substrate (panel I) or using ABC-VECTASTAIN Standard Kit and DAB Peroxidase Substrate Kit (panel II). Images of islets and two different ductal regions were taken before (A–C) and after (D–F) dehydration and coverslipping with Permount. The arrows point to the areas where staining was lost. Tissue: nPOD 6004-07. Scale bar: panels I and II, (A and D) 50 μm; panels I and II, (B, C, E and F) 100 μm.

Figure 4 Images of the adjacent pancreatic sections stained with either the monoclonal mouse anti-glucagon antibody using VECTASTAIN-ABC-Alkaline Phosphatase (AP) Standard Kit and Vector Red AP Substrate (panel I) or ABC-VECTASTAIN Standard Kit and DAB Peroxidase Substrate Kit (panel II). Images of islets and two different ductal regions were taken before (A–C) and after (D–F) dehydration and coverslipping with Permount. The arrows point to the areas where staining was lost. Tissue: nPOD 6004-07. Scale bar: panels I and II, (A and D) 50 μ m; panels I and II, (B, C, E and F) 100 μ m.

Figure 5 Images of the adjacent pancreatic sections containing glucagon-expressing neuroendocrine tumor stained with the monoclonal mouse anti-glucagon antibody using VECTASTAIN-ABC-Alkaline Phosphatase (AP) Standard Kit and Vector Red AP Substrate (panel I) or ABC-VECTASTAIN Standard Kit and DAB Peroxidase Substrate Kit (panel II). Images were taken before (A, B) and after (C, D) dehydration and coverslipping with Permount. Circles and arrows indicate the areas where staining was weakened or lost. Scale bar: panels I and II, (A and D) 50 μ m; panels I and II, (B, C, E and F), 100 μ m.

We report the novel finding that, when using AP-Vector Red as a substrate for staining of alpha-cells in human pancreatic sections, there was a disproportionately greater loss of color from the glucagon-positive cells present in the pancreatic ductal tissue compartment versus glucagon-positive cells resident within islets. This has important implications in the study of the pancreatic alpha-cell

population, both in terms of alpha cell distribution and turnover in healthy and diseased states.

Conclusion

In this report, we have shown that while DAB peroxidase was reliable for staining of alpha-cells, the use of AP-Vector Red is not a reliable method as the color is

partially washed out from alpha-cells in islets and completely lost from glucagon expressing cells in and around exocrine ducts during the dehydration step. This color loss can be overcome by using aqueous mounting medium or air drying prior to permanent mounting.

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Disclaimer Statements

Contributors TG and AB performed the experiments, contributed to study design and interpretation, and writing of the manuscript. PB contributed to the study design, study interpretation, and preparation of the manuscript.

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Conflicts of interest The authors declare that they have no conflict of interest.

Ethics approval Ethical approval was not required.

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