Detection techniques and treatment of pernicious anemia

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INTRODUCTION

Pernicious Anemia (PA) is also known as Biermer’s disease (Lahner & Annibale, 2009) and Addisonian anemia (Meecham & Jones, 1967). This auto-immune diseases is a macrocytic anemia arising due to the deficiency of Vitamin B12 (cobalamine) which in turn, in the presence of atrophic body gastritis (ABG), results in the deficiency of an intrinsic factor (a protein present in the bile juice) which is responsible for binding to Vitamin B12 and directing it towards ileum for absorption of cobalamine (Lahner & Annibale, 2009; Kulnigg-Dabsch, S. 2016; Toh et al., 1997). The presence of atrophic body gastritis initiates the destruction of oxyntic mucosa and therefore in the loss of parietal cells. Deficiency of the intrinsic factor occurs as a result of the destruction of parietal cells as they are responsible for the production of chlorhydric acid and the intrinsic factor (Toh et al., 1997 & Rode et al., 1986). Due to these events, antibodies synthesized by the body are directed towards the intrinsic factors and parietal cells thus making this disease an autoimmune disorder (antigens that initiate an immune response mimic that of body’s proteins resulting in autoimmune reaction).

The autoantibodies, especially the CD4+ T-cells, directed towards the parietal cells recognize the H+/K+-ATPase as the antigen. This ATPase enzyme, responsible for the secretion of gastric acid, belongs to the P-type ATPase family. The H+/K+-ATPase has a highly conserved 95kDa catalytic α subunit and 60-90kDa glycosylated β subunit. Due to a correlation between this autoimmune disease and anemia, and with the increase of anemia among people due to lifestyle among other factors, it is important to have

insights about the pathophysiology and mechanism of the disease that might help in the early detection, intervention and treatment (Lahner & Annibale, 2009; Kulnigg-Dabsch, S. 2016; Toh et al., 1997).

AIM

This practical aimed to provide a practical understanding for the diagnosis of pernicious anemia by demonstrating a reaction between the antibodies present in the patient sera and gastric proton pump antibody. This reaction was carried out using the techniques western blotting and immuno-peroxidase staining. The haematoxylin and eosin staining of the stomach sections of mouse will provide better understanding about the histopathology of the disease and therefore this was experimented by staining the sections of a healthy mouse and a mouse diagnosed with pernicious anemia.

OVERVIEW OF THE TECHNIQUES USED

One of the efficient ways to resolve proteins based on their molecular weight is SDS-PAGE. SDS-PAGE is the acronym for Sodium dodecyl sulfate (also known as Sodium Lauryl Sulfate) Polyacrylamide Gel Electrophoresis. SDS is a detergent that denatures the proteins into linear form. It also plays a role in decreasing the effect of structure of the protein such that the separation is entirely based on the molecular weight (length of the polypeptide chain). Polyacrylamide (cross linked polymer of Acrylamide and Bis-acrylamide) forms a matrix gel for the proteins such that smaller peptides migrate faster due to a lesser resistance from the matrix compared to that of high molecular weight proteins.

Western blotting is the technique used to detect the protein of interest based on its interaction with an antibody. The proteins are transferred onto a nitrocellulose membrane which is hydrophobic in nature. In the presence of buffer, wet transfer or semi-dry transfer of the proteins are carried out. Upon transfer on proteins onto the membrane, an antibody specific to the target protein (protein of interest) is probed after blocking the non-specific sites using Bovine Serum Albumin (BSA). The membrane is washed and treated with a secondary antibody that is tagged with enzymes such as Horse Radish Peroxidase (HRP). The activity of the enzyme based on its reaction with a known substrate is used to detect the presence of the target protein that can detected by a chemiluminescent or chromogenic method.

Hematoxylin and Eosin (H&E) staining is the most commonly used staining techniques for understanding the histopathology of the tissue segments. Eosin is an acidic dye which means that the dye is negatively charged and therefore stains components that are basic in nature, such as the cytoplasm. On the contrary, hematoxylin, generally referred to as basic dye, is positively charged and thus stains components that are acidic in nature such as the nucleus of cells. Hematoxylin is a combination of hematein dye along with Aluminium ions (Al3+). The aluminium ions present in the mordant salts used along with dye binds to the tissue followed by the binding of the stain to the ions forming a tissue – mordant – hematoxylin linkage. The eosin dye stains the basic components pink and the hematoxylin dye stains the components bluish purple.

Staining of the cells can be carried out specifically to visualize the target protein. This can be achieved by allowing the antigen to react with an antibody conjugated to enzymes such as horse radish peroxidase. This enzyme upon reaction with the substrate will produce either a chemiluminescent or a chromogenic result that is based on the substrate. Cells within a tissue are incubated along with a primary antibody. Secondary antibody directed against the primary antibody is allowed to react with the cells containing primary antibody. The secondary antibody is conjugated along with HRP enzyme. The usage of two types of antibodies increases the specificity of the reaction and visualization of the target protein. Commonly used immunoperoxidase staining techniques include biotin streptavidin immunoperoxidase (B-SA).

METHODOLOGIES

SDS-PAGE OF PROTEINS DERIVED FROM MOUSE STOMACH CELLS

The protein extracted from the mouse stomach cells were diluted to a final concentration of 2µg/µL. To 200µL of the protein sample 50µL of 5x SDS-sample buffer was added. A precast polyacrylamide gel was placed into the electrophoresis tank whose reservoir was filled with Tris-Glycine buffer (running buffer). Protein molecular weight marker ranging from 10kDa to 250kDa was loaded into the first well followed by positive control and negative control in the second and third wells respectively followed by 25µL into each of the other wells. The apparatus was connected to the power supply and was run at 200V until the yellow (10kDa band) reached the

bottom of the gel. The gel was removed from the cassette and was placed into transfer buffer.

WESTERN BLOTTING

The transfer of proteins was carried out by placing one ultra-thick filter paper at the bottom onto which the nitrocellulose membrane was placed with the label facing up. The SDS-PAGE gel was aligned on top of the nitrocellulose membrane followed by one piece of ultra-thick filter paper. This stacking was carried out inside Turbo-blot cassette and the transfer was allowed to happen at 25V for 7 minutes. The nitrocellulose membrane, after the transfer was completed, was placed inside a container containing 50mL of 0. 1% Ponceau stain. This is useful for detecting the protein bands on the nitrocellulose membrane as the dye form a red stain upon reaction with the protein bands.

Upon visualization of the band, the lanes were marked with a pencil and the membrane was washed with 50mL of 0. 1M NaOH until the stain disappeared. The membrane was then washed in 50mL of TBS buffer. To restrict the non-specific binding of proteins, 50mL of blocking solution, prepared by adding 5% skim milk powder to TBS, was added to the membrane.

The strips were then placed onto a piece of parafilm and 100µL of human sera sample was added onto the strip which was then covered by another parafilm to prevent it from drying. The reaction between autoantibodies in human sera and antibodies in mouse protein was allowed to happen at room temperature for one hour. The strips were then washed thrice with TBST and

rocking for 10 minutes. The strips were then placed onto a fresh parafilm and 100µL of secondary antibody, raised in sheep, tagged with horse radish peroxidase enzyme, directed against human antibody was added and incubated for 45 minutes. The strips were then washed thrice using TBS for 5 minutes with rocking. Before the visualization of strip, 4mL of freshly prepared Lumi-Light substrate was added and incubated for 5 minutes. The strips were visualized using Chemidoc apparatus and captured.

Hemotoxylin and Eosin Staining

The stomach tissue sections of mouse fixed with formalin and embedded with paraffin were stained using H&E staining. The pre-washed slides were placed in hematoxylin stain for 2 minutes and rinsed with tap water to remove excess dye. The slide was then dipped into 1% acid alcohol solution for 3 seconds followed by immediate washing with water. The slides were incubated in Scott’s tap water for 30 seconds followed by placing it in eosin Y stain for 4 minutes and the excess stain was blotted onto a tissue. The slide was then placed in 90% ethanol for 30 seconds and the excess liquid was drained followed by placing it in 100% ethanol for 30 seconds and blotting the excess liquid. The slides were then incubated again in 100% ethanol for 2 minutes followed by air dry to evaporate the ethanol. A drop of Organolimoline mounting medium was added on the section and covered with a cover slip. The slides were then examined under the microcope using 40x magnification and 100-400x magnification. The diagrams were then recorded.

Immunoperoxidase staining

Slides washed in histoclear solution and then with ethanol were transferred to a Sequenza coverplate clip and inserted into the Sequenza rack. 200µL of 0. 3% peroxide (H2O2) was added and incubated for 30 minutes at room temperature to remove endogenous peroxidase activity. The slides were then washed with PBS and 200µL of 1% BSA in PBS was added to restrict the non-specific binding of the proteins. 200µL of patient's serum sample was added inside the space between the slide and cover plate. This was incubated at 4o overnight. The slides were then washed with PBS and 200µL of the sheep anti-human Ig HRPO conjugate (1: 300 diluted) was added onto the section and incubated for 45 minutes. After washing the slides with PBS, 200µL of DAB substrate was added and incubated for 5-10 minutes. The slide was removed from the rack and dipped into hematoxylin. Upon washing with ethanol and drying, a drop of Organolimolene mounting medium was added on to the section and covered with coverslip. The slide was observed under the microscope.

RESULTS

The protein extracted from mouse stomach cells were separated using SDS-PAGE and was transferred onto membrane for western blotting. Upon visualization using Chemidoc, it was observed that patient serum sample 1, 3, and 5 had positive result. The lane corresponded to the positive control and the molecular weight was estimated to be 75kDa corresponding to the β subunit of the proton pump.

The cells were visualized using H&E staining and the patients' sample 1, 3, and 5 corresponded to the cells visualized in positive control.

DISCUSSION

Based on the obtained results, it was inferred that patients' sample 1, 3, and 5 were diagnosed with pernicious anemia. This was due to the fact that the western blot showed a blot on the lane corresponding to these samples and to the positive control. Positive control here refers to sample diagnosed with pernicious anemia and negative control refers to the sample derived from healthy individual. The antibodies were directed against the particular species and therefore, it can be inferred that the reaction happened was between the autoantibodies in the sera and H+/K+-ATPase in the mouse (Jeffries & Sleisenger, 1965).

Also, upon staining, the histopathology of the sections were observed. Based on the sections, the loss of parietal cells were observed along with decrease in the width between the gastric pits. The gaps in the sections of the positive control sample indicates complete loss of parietal cells in that region. Samples diagnosed with pernicious anemia was also found to have inflammation in the gastric pit regions thereby decreasing the gaps between them (Toh et al, 1997 & Dholakia et al., 2005). Same samples were found to be positive for immunoperoxidase staining indicating the reaction between autoantibodies and the ATPase enzyme.

Apart from these techniques, direct ELISA can also be performed to confirm the reaction between the antigen (ATPase) and autoantibodies which will also quantify the concentration of protein present in the sample.