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Original Article

3-Layer Immunoperoxidase Protocol Reaction, Endomucin, and Alpha-smooth Muscle Actin Detection

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ABSTRACT

Background and objectives. *Immunohistochemistry (IHC) is the detection of antigens in tissue sections by specific antibodies. It has the unique advantage over other methods for detection proteins like α-Smooth Muscle Actin and endomucin, enabling the correlation of antigens with their location within a tissue. The aim of the study was to identify α-SMA and endomucin in cardiac muscle tissue and arterial blood vessels which have important diagnostic purposes.* ***Methods****. Three Specimens (aSMA, Endomucin and control) of formalin-fixed paraffin embedded mouse embryo tissue sections have been de-waxed, rehydrated. Then they were covered with accurate primary antibody: αSMA slide in dilute mouse monoclonal anti-smooth muscle actin, Endomucin slide in dilute rat monoclonal anti-endomucin and the control slide gets just PBS (no-primary control). Then the sections were covered with the right secondary antibody: αSMA slide with biotinylated rabbit anti-mouse IgGIgG diluted and Endomucin slide with biotinylated rabbit anti-rat IgG, control slide with either antibody. Next color reagent was applied; it contains 3,3'-Diaminobenzidine and 0.3% hydrogen peroxide. Finally examine slides using a microscope.* ***Results****. The results showed that there was different brown 3,3'-Diaminobenzidine staining patterns in the two test slides for the individual primary antibodies. The 3,3'-Diaminobenzidine Staining was highly expressed in the external part of the section as a result of the presence of α-Smooth Muscle Actin. Whereas, in case of Endomucin, the stain is expressed in the central part of specimens due to presence of the endothelial tissue. and no staining in the primary control sections.* ***Conclusion****. As a result of the presence of α-Smooth Muscle Actin in the muscular tissue,3,3'-Diaminobenzidine Staining was highly expressed in the external part of the section. However, in case of Endomucin the stain is expressed in the central part of specimens due to presence of the endothelial tissue.*

Keywords*: Immunohistochemistry, Antigens, Antibodies, Endomucin, Actin.*

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INTRODUCTION

Immunohistochemistry

(IHC) is the detection of antigens in tissue sections by specific antibodies. It is a good technique in many laboratories for diagnostic and research purposes and it has the unique advantage over other methods for detection proteins like α-Smooth Muscle Actin and endomucin, enabling the correlation of antigens with their location within a tissue. It is used in the diagnosis of atypical cells like those found in cancerous tumors as a result of the presence of specific markers of cell phenotype which are useful in the diagnosis of lymphomas and other tumor types. IHC was introduced first by Coons in the late

1940s and, since then it has been applied extensively to multiple areas of fields of cell and tissue biology, embryology, and pathology [1]. There are two methods used for the immunohistochemical detection of antigens in the tissue section, the direct and the indirect methods.

The direct method (figure1) uses one labeled antibody, which binds immediately to the antigen being targeted. It is a simple and fast protocol. However, it can undergo tribulations with sensitivity due to the limited signal amplification as Signals might fade during the study and is consequently not used as much as the indirect technique [2].

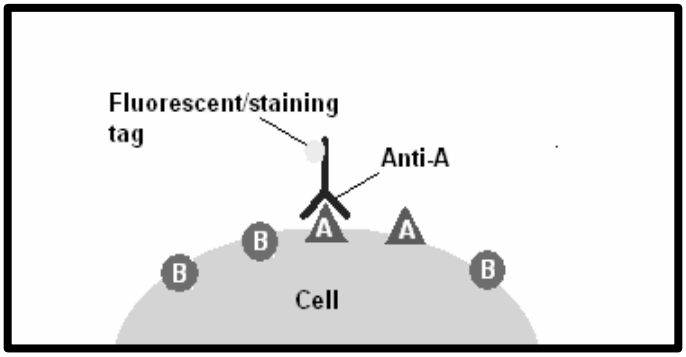


Figure 1. The direct method of immunohistochemistry uses one labeled antibody, which binds straight to the antigen being stained for. Image taken from www.websters-online-dictionary.org/images/wik

The indirect method (figure2) involves an unlabelled primary antibody which reacts with the tissue antigen, and a labeled secondary antibody which reacts with the primary antibody. This procedure is more sensitive since there is a signal amplification process as a number of secondary antibodies can bind to each primary antibody at different sites. Visualizing an antibody-antigen interaction can be done in a number of ways. In the most common example resume, an antibody is conjugated to an enzyme, like peroxidase, that can catalyze a color-producing reaction.

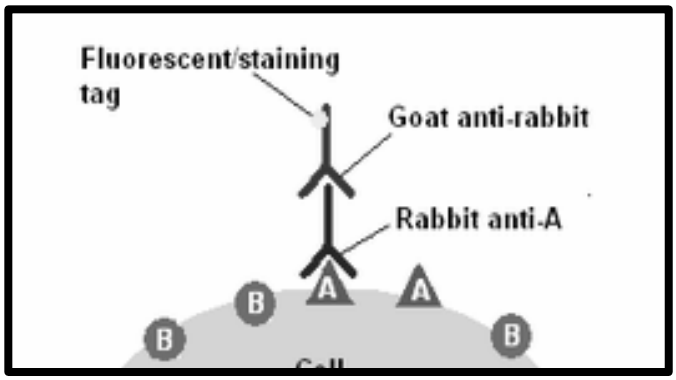


Figure 2. The Representation of the indirect method of immunohistochemistry uses one antibody against the antigen being probed for, and a second, labeled, antibody against the first. Image taken from www.websters-online-dictionary.org/images/wik

A fast and simple indirect immunoperoxidase staining technique is used in many laboratories to demonstrate proteins in IHC procedures. Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein or rhodamine[3]. The aim of the study was to identify α-SMA and endomucin in cardiac muscle tissue and arterial blood vessels in mouse embryos tissue by using indirect IHC method which has important diagnostic purposes.

Alpha-smooth muscle actin (SMA)

Alpha-smooth muscle actin (SMA), An actin isoform that contributes to cell-generated mechanical tension, is normally restricted to cells of vascular smooth muscle, but SMA can also be expressed in certain non-muscle cells, most notably myofibroblasts. These cells are present in healing wounds, scars, and fibro contractive lesions where they contribute to fibrosis [4]. In myofibroblasts, cell-generated traction forces associated with SMA contribute to matrix remodeling, but exogenous mechanical forces can also increase SMA expression.

Endomucin

Endomucin is a protein that in humans is encoded by the EMCN gene. Endomucin is a marker for

endothelial cells. It is an endothelial sialomucin that was recently identified with the help of monoclonal antibodies raised against mouse endothelial cells. Cloning of human endomucin allowed us to generate monoclonal antibodies against soluble recombinant forms of human endomucin [5].

METHODS

Preparation of the tissue

Three Specimens of formalin-fixed paraffin embedded mouse embryo tissue sections have been de-waxed, rehydrated through an ethanol series, and had endogenous to peroxidases blocked (2.5% H2O2 in methanol) which can be found in normal erythrocytes.

Primary Antibody covering

Three slides were labeled with primary Ab name (1 x SMA, 1 x Endo, 1 x No-primary antiserum control). To decrease non-specific binding of antibodies, each specimen was covered with 200µl of (pre-diluted rabbit serum). After 10 minutes excess was eliminated without washing.Then sections were covered with accurate primary antibody: ⟨SMA slide in 200µl diluted primary antibody (monoclonal anti-smooth muscle actin diluted 1:4,000 in Phosphate Buffered Saline) Endomucin slide in 200µl primary antibody (rat monoclonal anti-endomucin diluted 1:250 in PBS) control slide gets just PBS (no-primary control).

Secondary antibody covering

After 20 minutes of incubation and washing all slides in PBS for 2 minutes x3 changes. The sections were coverd with the right secondary antibody: αSMA slide with 200µl of biotinylated rabbit anti-mouse IgG diluted 1:250 in PBS and Endomucin slide with 200µl biotinylated rabbit anti-rat IgG diluted 1:200 in PBS, control slide with either D or E. Keep covered moist for 15 minutes.After Washing all slides in PBS for 2 minutes x3. All slides were incubated in tertiary layer streptavidin-peroxidase diluted 1:500 in PBS for 15 minutes. Then Washed in PBS for 2 minutes x3.

3,3′-Diaminobenzidine staining

Next 200µl of color reagent were applied ; it contains 3,3′-Diaminobenzidine and 0.3% hydrogen peroxide, observed carefully over 2 – 4 minutes and washed in PBS before background brown staining appears. PBS several times was washed with counterstained with haematoxylin for 2 minutes, then washed in tap water then dehydrated in graded alcohols (60%, 80%, 100%) 2 minutes each using the plates. Clear in Xylene for 2 minutes, and after that dry around the tissue slice do not let the slice to be dry. Mount in DPX.Finally examine slides using a microscope (shown in figure3).

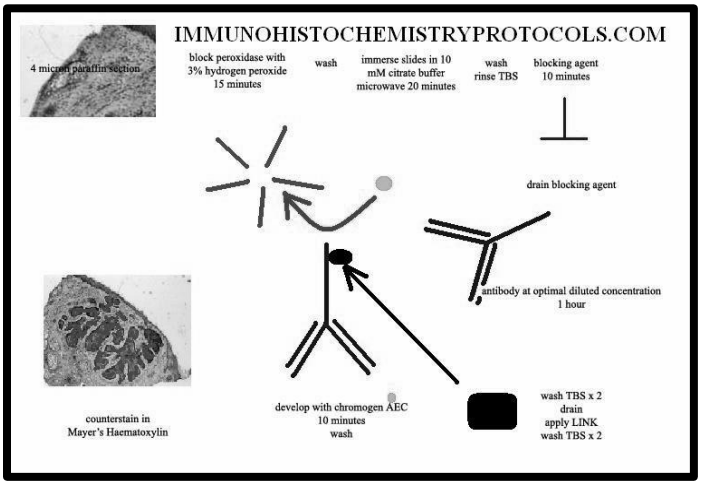


Figure3. Representation of immunoperoxidase protocol Image taken from www.websters-online-dictionary.org/images/wik

Ethical approval

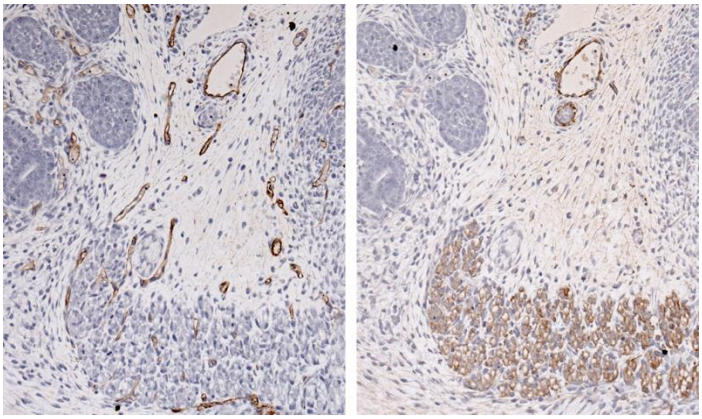
All experimental procedures and animal maintenance were performed according to the bioethical research guide established by the Libyan National Committee for Biosafety and Bioethics. Laboratory animals and sample collection Male 14 days albino mice were bred in the animal house of the Zoology department, Faculty of Science, University of London, UK. Mice were housed in plastic cages containing wooden flakes in an airconditioned room. They were kept under standard laboratory conditions (24 to 26^{0}C, and 55 to 60% humidity) with a 12-hour light/dark cycle, and fed standard commercial laboratory chow. Water and

standard pellet diets were available ad libitum throughout the experimental period.

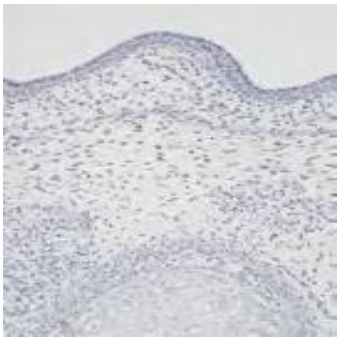
RESULTS AND DISCUSSION

Light Microscopy

There was different brown 3,3′-Diaminobenzidine staining patterns in the three test slides for the individual primary antibodies. As result of presence α-Smooth Muscle Actin in the muscular tissue, 3,3′-Diaminobenzidine staining was highly expressed in external part of section whereas in case of Endomucin the stain expressed in the central part of specimens due to presence the endothelial tissue and no staining in the no-primary control section.

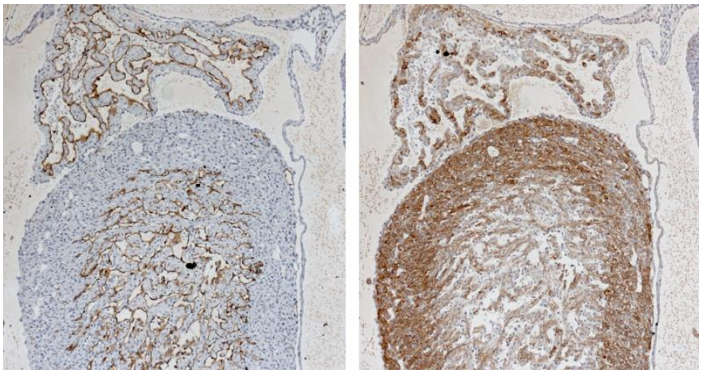


Endomucin *αSMA*



Control

Figure4. A Rapid Protocol for Immunohistochemistry in 14d mouse embryo (arterial blood vessels)



Endomucin *αSMA*

Figure5.14d mouse embryo: heart (αSMA is expressed before cardiac actin)

Both cardiac and smooth muscle fibers varied in their expression of actin isotopes, specifically recognizing the single α-isoform feature of smooth muscle cells and those cells with myofibroblastic differentiation. Antibodies to α-SMA are used in a number of diagnostic situations [6]. These include the detection of myoepithelial cells, which are admixed; with epithelial cells in benign proliferative lesions that affect the breast and distinction from neoplastic proliferations. Myoepithelial cells plus line benign ductules of the breast were compared to their absence in neoplastic tubules [7]. Furthermore, α-SMA helps predict likely outcomes in breast cancer patients. α-SMA is a marker that allows recognition of myofibroblastic differentiation and has been used in studies of idiopathic pulmonary fibrosis [4]. α-SMA is used mostly as a discriminator of smooth muscle tumors in the identification of spindled and pleomorphic tumors [8]. It was important to highlight that this marker should not be used in separation because myogenic determinants are not constantly created by normal and neoplastic cells simultaneously, the maximum analytical yield is gained with a group of antibodies that include α-SMA, desmin and muscle-specific actin [9]. Smooth muscle

alpha actin is of future interest because it is one of a small number of genes whose expression is fairly limited to vascular smooth muscle cells. Whereas Endomucin is a potential cell surface marker [10]. Human endomucin is predicted to determine a 261-aa, 27.5-kDa protein by a transmembrane sequence and multiple glycosylation sites.

Several studies have been conducted to detect the two protein expressions in blood vessels and cardiac muscle tissue. According to Nungki Anggorowati,Chatarina Ratna Kurniasari, Karina Damayanti[11] Alpha-smooth muscle actin (α-SMA) is an isoform of actin, positive in myofibroblasts and is an epithelial to mesenchymal transition (EMT) marker. EMT is a process by which tumor cells develop to be more hostile and able to metastasize collagen and α-SMA area fractions in stroma were higher in benign than in malignant neoplasms in the two tissues [12]

CONCLUSION

The present study showed that α-Smooth Muscle Actin presented primarily in the muscular tissue while the human endomucin expression is mainly, if not exclusively, endothelial cell-specific. Human endomucin appears abundantly in highly vascular tissues such as heart, kidney, and lung. Moreover "Immunohistochemistry revealed that the endomucin-1 transcript was specifically expressed in the endothelial cells of dorsal aorta of E10.5 mouse embryo. Overexpression of endomucin-1 strongly inhibited adhesion and aggregation of cells, including cultured endothelial cells from E10.5 dorsal aorta. These data suggest that endomucin-1 may play a role in detachment of hematopoietic cells from endothelium during early haematopoiesis" [7]

Disclaimer

The article has not been previously presented or published, and is not part of a thesis project.

Conflict of Interest

There are no financial, personal, or professional conflicts of interest to declare.

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