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Original Article

The comparison of methods to identify the presence of fibrocytes in formalin-fixed, paraffin-embedded archival cardiac tissue with coronary heart disease☆

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ABSTRACT

The purpose of this study was to find the optimal technical approach to identify the presence of fibrocytes in formalin-fixed, paraffin-embedded archival cardiac tissue with CHD (coronary heart disease). Using the coexpression markers CD45 and αSMA, the presence of fibrocytes was examined by three different methods, including double immunohistochemistry staining, combination labeling of immunohistochemistry and immunofluorescence and double immunofluorescence labeling. Double immunohistochemistry staining was very difficult to identify the $CD45^+/\alpha SMA^+$ fibrocytes. Although combination staining of immunohistochemistry and immunofluorescence has made it possible to evaluate the co-localization of CD45 and αSMA in the fibrocytes, this method was prone to produce many false positive cells. In contrast, $CD45^+/\alpha SMA^+$ fibrocytes could be clearly recognized by double immunofluorescence labeling. In conclusion, double immunofluorescence labeling is the optimal technical approach to identify the presence of fibrocytes in routinely processed cardiac tissue with CHD.

Introduction

Fibrocytes are the unique mesenchymal progenitor cells found to be an important source of fibroblasts and myofibroblasts during tissue remodeling and fibrosis. They coexpress the common leukocyte antigen CD45, the hematopoietic stem cell antigen CD34, and mesenchymal markers such as collagen I, fibronectin and alpha smooth muscle actin (αSMA) [1–4]. Since the classic markers for fibrocytes are CD45, CD34, Col I and αSMA, there are three double staining methods used to identify the presence of fibrocytes in paraffin-embedded tissue, including double immunohistochemistry staining, combination labeling of immunohistochemistry and immunofluorescence and double immunofluorescence labeling [5–8]. Using the coexpression markers of CD45 and αSMA, the three methods were compared with each other in formalin-fixed, paraffin-embedded archival cardiac tissue with CHD (coronary heart disease) to choose the optimal technical approach to identify the presence of fibrocytes.

Materials and methods

All of the paraffin sections are 5 μm thick, obtained from 10 formalin-fixed, paraffin-embedded archival human cardiac tissue blocks with CHD. Remove paraffin from sections by immersing slides in two changes of xylene, 5 min each. Hydrate in two changes of 100% ethanol for 5 min each, followed by 95%, 90%, 80% and 70% ethanol for 3 min each. Rinse in distilled water for 3 min, and then rinse with PBS for 3× 5 min. These dewaxed and rehydrated sections were subjected to the following three methods.

Double immunohistochemistry staining

We used Double Immunohistochemistry Kit with peroxidase and alkaline phosphatase detection systems (Maixin Technology Co. Ltd., Fuzhou, China) to examine the presence of fibrocytes. Immunohistochemistry staining combined the mouse CD45

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antibody (BD Biosciences, Pharmingen, Leiden, Netherlands) and mouse αSMA antibody (Millipore, USA). To evaluate fibrocytes, 10 randomly chosen areas of per case were analyzed by light microscopy. Slide processing was performed according to the instructions as follows: incubate the sections in 10-mM citrate buffer (pH 6.0) in a pressure cooker for 1.5 min, rinse with PBS for 3× 5 min. Outline sections with a hydrophobic wax pen to avoid antibody spreading and possible drying. Incubate sections with endogenous peroxidase blocking solution for 10 min at room temperature. Rinse with PBS for 3× 5 min. Incubate with non-immune serum for 10 min at room temperature. Incubate with the mouse CD45 antibody (20 μg/ml) (BD Biosciences, Pharmingen, Leiden, Netherlands) overnight at 4 °C. Rinse with PBS for 3× 5 min. Incubate with biotinylated second antibody at room temperature for 10 min. Rinse with PBS for 3× 5 min. Incubate with streptavidin alkaline phosphatase antibody at room temperature for 10 min. Rinse with PBS for 3× 5 min. Incubate with a chromogenic agent of alkaline phosphatase (BCIP/NBT) for 15 min at room temperature until color develops. Monitor color development on wet slides by light microscopy. Wash twice with distilled water, and then rinse with PBS for 3× 5 min. Incubate with a reinforcing agent of double staining at room temperature for 10 min. Rinse with PBS for 3× 5 min. Incubate with non-immune serum for 10 min at room temperature. Incubate with the mouse αSMA antibody (dilution 1:100) (Millipore, USA) for 1 h at room temperature. Rinse with PBS for 3× 5 min. Incubate with biotinylated second antibody at room temperature for 10 min. Rinse with PBS for 3× 5 min. Incubate with streptavidin peroxidase at room temperature for 10 min. Rinse with PBS for 3× 5 min. Incubate with a chromogenic agent of peroxidase (AEC) for 8 min at room temperature until color develops. Monitor color development on wet slides by light microscopy. Wash in water, counterstain in hematoxylin, and rinse with PBS. Cover slip with aqueous-based medium (ClearMount).

Combination labeling of immunohistochemistry and immunofluorescence

Double labeling using a combination of immunohistochemistry and immunofluorescent probes was performed to identify fibrocytes coexpressing cell markers CD45 and αSMA in paraffin-embedded heart samples. Briefly, slides were processed in a standard fashion as previously described [5]. The sections were incubated in 10-mM citrate buffer (pH 6.0) in a pressure cooker for 1.5 min. The tissues were incubated with non-immune serum for 10 min at room temperature. Tissues were incubated with mouse CD45 (20 μg/ml) (BD Biosciences, Pharmingen, Leiden, Netherlands) overnight at 4 °C. The signal of CD45 was amplified using biotinylated goat anti-mouse antibody (Maixin Technology Co. Ltd., Fuzhou, China), and followed by streptavidin peroxidase (Maixin Technology Co. Ltd., Fuzhou, China). The resulting chromogen was detected using diaminobenzidine-hydrogen peroxide (DAB Map kit, Maixin Technology Co. Ltd., Fuzhou, China). The tissues were incubated with non-immune serum for 10 min at room temperature. The tissues were then incubated with primary antibodies of anti-αSMA (dilution 1:25) (Millipore, USA) for 1 h at room temperature, followed by biotinylated anti-mouse antibody (Maixin Technology Co. Ltd., Fuzhou, China). For visualization of the biotinylated antibody, we used Alexa Fluor 594 conjugate (20 μg/ml) (Molecular Probes, Eugene, OR). Nuclei were stained with DAPI (Boster Biotechnology Co. Ltd., Wuhan, China). Controls were performed with/without primary antibody or with/without secondary antibody on CHD tissues, and were included in all experiments to correct for background.

Images were taken of Leica DM2500 microscope using light microscopy to visualize the CD45 and fluorescent microscopy at the 590 and 360 wavelengths to assess fluorescent staining of αSMA and nuclei. The images of immunohistochemistry staining and immunofluorescence labeling at the same field were then merged by Leica DM2500 microscope. To evaluate fibrocytes, 10 randomly chosen areas per case were analyzed, and after optical sectioning, triple stained spots were regarded as positive hits.

Double immunofluorescence labeling

The sections were incubated in 10-mM citrate buffer (pH 6.0) in a pressure cooker for 1.5 min. The tissues were incubated with non-immune serum for 10 min at room temperature. Tissues were incubated with mouse CD45 (20 μg/ml) (BD Biosciences, Pharmingen, Leiden, Netherlands) overnight at 4 °C. The signal of CD45 was amplified using biotinylated goat anti-mouse antibody (Maixin Technology Co. Ltd., Fuzhou, China). For visualization of the biotinylated antibody, Alexa Fluor 488 conjugate (20 μg/ml) (Molecular Probes, Eugene, OR) was used. The tissues were incubated with non-immune serum for 10 min at room temperature. The tissues were then incubated with primary antibodies of anti-αSMA (dilution 1:25) (Millipore, USA) for 1 h at room temperature, followed by biotinylated anti-mouse antibody (Maixin Technology Co. Ltd., Fuzhou, China). For visualization of the biotinylated antibody we used Alexa Fluor 594 conjugate (20 μg/ml) (Molecular Probes, Eugene, OR). Nuclei were stained with DAPI (Boster Biotechnology Co. Ltd., Wuhan, China). Controls were performed with/without primary antibody or with/without secondary antibody on CHD tissues, and were included in all experiments to correct for background. For fluorescence analyses, Leica DM2500 microscope or Olympus FV1000 confocal microscope was used. Sequential scanning for the different fluorophores was performed, and the images were then merged. To evaluate fibrocytes, 10 randomly chosen areas of per case were analyzed, and after optical sectioning, triple stained spots were regarded as positive hits [6].

Results

Using the coexpression markers CD45 and αSMA, the three double staining methods, including double immunohistochemistry staining, combination labeling of immunohistochemistry and immunofluorescence and double immunofluorescence labeling, were compared with each other in 10 formalin-fixed, paraffin-embedded archival cardiac tissues with CHD to choose the optimal technical approach to identify the presence of fibrocytes.

Although the general architecture of the cardiac tissue was well preserved, CD45 was clearly expressed in cytomembrane and αSMA was definitely expressed in the cytoplasm in all cardiac tissues, double immunohistochemistry staining was very difficult to identify the $CD45^+/\alpha SMA^+$ fibrocytes (Fig. 1). In addition, combination staining of immunohistochemistry and immunofluorescence has made it possible to evaluate the co-localization of CD45 and αSMA in the fibrocytes, but because the $CD45^+/\alpha SMA^+$ fibrocytes were purple or white and were difficult to distinguish from the other cellular nuclei and the white background, this method was prone to produce many false positive cells (Fig. 2). In contrast, $CD45^+/\alpha SMA^+$ fibrocytes could be clearly recognized by double immunofluorescence labeling (Fig. 3).

Discussion

Immunostaining is used to localize specific antigens in tissues with labeled antibodies based on the specific antigen-antibody reaction. The immune reactive products can be visualized by markers such as fluorescent dye, enzyme, radioactive element and colloidal gold. Theoretically, immunostaining can identify any