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Immunohistochemical staining of heparan sulfate (HS) and collagen type XVIII (col18) core proteins in islet beta cells of formalin-fixed human pancreas and isolated human islets V.3

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We use this protocol and it's working

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Abstract

Paraffin sections (4 µm thickness) of formalin-fixed human pancreases and isolated human islets were treated with 0.05% pronase for antigen retrieval. HS and Col18 HSPG core proteins were detected immunohistochemically using 10E4 anti-HS (US Biological/Amsbio) and anti-Col18 (Santa Cruz), respectively, with horseradish peroxidase-conjugated rabbit anti-mouse Ig (Dako). Background staining was checked using the corresponding isotype control Ig instead of the primary antibody. 3-amino-9-ethylcarbazole (AEC) was used as the chromogen. For morphometry, stained sections were imaged using a light microscope with attached camera (Olympus BX41). Image J software with color deconvolution plugin was used for the quantitative analysis of the % of islet area stained

Guidelines

10E4 anti-heparan sulfate (HS) mAb identifies highly sulfated HS localised in human beta cells but does not identify the less sulfated HS in alpha cells.

Reference:
Theodoraki A, Hu Y, Poopalasundaram S et al (2015) Mol Cell Endocrinol 399: 296-310.

Before start

Materials:

1. Prepare graded alcohols and xylene for deparaffinizing tissue sections: 2 x xylene (250 ml/slide container), 2 x absolute ethanol (250 ml/slide container), 1 × 90% ethanol (250 ml), 1 × 70% ethanol (250 ml)

2. Prepare acetate buffer components:
(i) 0.1N acetic acid: 290 µl glacial acetic acid in 50 ml deionized water
(ii) 0.1M sodium acetate: 410 mg anhydrous CH_3COONa in 50 ml deionized water.
Prepare 0.1M acetate buffer (pH 5.2) by mixing 10.5 ml 0.1N acetic acid and 39.5 ml 0.1M sodium acetate.

3. Prepare stock solution of 3-amino-9-ethylcarbazole (AEC; chromogen, 8 mg/ml: 40 mg AEC in 5 ml N-N-dimethyl formamide; protect from light and refrigerate at 4°C.

4. Prepare M.O.M. diluent: 200 µl M.O.M. protein concentrate stock solution (M.O.M immunodetection kit) in 2.5 ml phosphate-buffered saline (PBS) for use either as a blocking step to minimize non-specific Ig binding or for diluting antibodies.

5. Mabs and pAbs:
10E4 (mouse anti-human HS) mAb, Amsbio #370255-1
Mouse anti-mouse collagen type XVIII (Col18A1), Santa Cruz Biotechnol #1837-46
Horseradish peroxidase (HRP) -conjugated rabbit anti-mouse Ig, Dako #PO161 (alternatives: HRP-rabbit anti-mouse IgM, Thermo Fisher #31456 (for HS); HRP-rabbit anti- mouse IgG (H+L), Thermo Fisher#31450)
Mouse IgM_K , BD Biosciences #550340
Mouse IgG_{2bK} , BD Biosciences #557351

6. Other reagents:
Hydrogen peroxide (30% w/w), Chem-Supply Pty Ltd (Australia) #HA154-500M
Methanol, Merck #106009
Pronase, Calbiochem #537088
3-Amino-9-ethylcarbazole (AEC), Sigma-Aldrich #A5754
Animal free blocker, Vector Labs #SP-5030
Stock protein concentrate, M.O.M immunodetection kit, Vector Labs # PK-2200
N-N-dimethyl formamide, Sigma #D158550
Glycergel mounting medium, Dako #C0563

1 See Guidelines, "Before starting".

2 Deparaffinize slides in each xylene for 1 min. rehydrate slides in graded alcohols beginning in absolute ethanol (10 dips)/ container of absolute ethanol), followed by 90% ethanol (10 dips) and 70% ethanol (10 dips). Wash well in running tap water for 5 min.

3 Blot around sections with a tissue wipe, encircle the sections using a diamond pencil and place in a slide container of tap water (250 ml).

4 Block endogenous peroxidase activity by incubating sections in 3% hydrogen peroxide in methanol (25 ml 30% H_2O_2 + 225 ml methanol).

5 Wash 2 × 2 min in 250 ml phosphate-buffered saline (PBS) followed by wash in running tap water for 4-5 min.

6 Prewarm slide tray containing low level of water (to humidify) in 37°C incubator.

7 Prepare 0.5 mg/ml (0.05%) pronase (#537088 Calbiochem; for antigen retrieval to expose HS epitopes): 2.5mg pronase in 5 ml deionized water.

8 Wipe around sections using tissue and cover each section with pronase solution. Incubate sections in a humidified slide tray at 37°C (incubator) for 10 min.

9 Wash slides for 2 × 2 min in 250 ml PBS.

10 Wipe around sections using tissue. Block non-specific binging of Ig:
(i) For HS immunostaining, apply animal free block (diluted to 20% v/v with deionized water) to tissue sections and incubate for 5 min at room temperature.
(ii) For Col18 immunostaining, apply diluted protein concentrate and incubate for 5 min at room temperature.

11 Tip off excess block in Step 10(i) or 10(ii), wipe around sections using tissue and incubate with 0.2 mg/ml anti-HS mAb (or 0.2 mg/ml mouse IgM as isotype control; diluted in protein concentrate solution) and incubate for 1 hour or incubate with 2-4

µg/ml anti-col18 mAb (or 2-4 µg/ml mouse IgG_{2bk} as isotype control; diluted in protein concentrate solution), 125-150 µl/section at room temperature for 30 min.

12 Wash off primary antibody with PBS and transfer slides to slide container with 250 ml PBS. Wash 2 × 2min.

13 Wipe around sections using tissue and incubate with 26 µg/ml secondary HRP-rabbit anti-mouse Ig, 130-150 µl/section, for 30 min at room temperature. (Alternatives: 3.2 µg/ml secondary HRP-rabbit anti-mouse IgM (for HS); 3.2-6.4 µg/ml secondary HRP-rabbit anti-mouse IgG (for Col18)).

14 Wash off secondary antibody with PBS and transfer to slide container with 250 ml PBS. Wash slides 2 × 2min.

15 Prepare AEC working solution: 4.75 ml acetate buffer (see Guidelines), 0.25ml AEC stock solution and 25 µl 3% H_2O_2. Filter using a disposable 0.2 µm filter. Use within 2 hours of preparation, refrigerate for short-term storage. Protect from light.

16 Wipe around sections using tissue and cover the sections with AEC solution for 30 min at room temperature.

17 Wash off AEC solution with deionized water and transfer slides to slide container with 250 ml deionized water. Wash 3x in 10min.

18 Lightly counterstain with Gill's hematoxylin, wash in deionized water (2 x) and briefly dip in ammonium water (100 µl ammonia in 250 ml deionized water), 2 × 2 sec. Wash in deionized water (2 x in 250 ml) and coverslip using glycergel mounting medium.

19 Image sections using a light microscope with camera attachment. Use Image J software with color deconvolution plugin to determine % of islet area stained.