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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.2

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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

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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 STARTING

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 x 90% ethanol (250 ml), 1 x 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 CH₃COONa 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 Ig_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 Wipe around sections with a tissue, 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% H₂O₂ + 225 ml methanol).
- 5 Wash 2 x 2 min in 250 ml phosphate-buffered saline (PBS) followed by wash in running tap water for 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 x 2 min in 250 ml PBS.
- 10 Wipe around sections using tissue. Block non-specific binding 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 9(i) or 9(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 µg/ml anti-col18 mAb (or 2 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 x 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 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 x 2min.

Prepare AEC working solution: 4.75 ml acetate buffer (see Guidelines), 0.25ml AEC stock solution and 25 µl 3% H₂O₂.

- 15 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 x 2 sec. Wash in deionized water (2 x in 250 ml) and coverslip using glycerol 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.