VERSION 2
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Immunohistochemical staining of CD44 core proteins in islet beta cells of formalin-fixed mouse pancreas V.2

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Lora Starrs[1], Debra Brown[1], Sarah Popp[1], Charmaine Simeonovic[1]

[1]The Australian National University

Charmaine Simeonovic

ABSTRACT

Paraffin sections (4mm thickness) of formalin-fixed mouse pancreases were treated with heat/citrate buffer for antigen retrieval. CD44 core proteins were detected immunohistochemically using rat anti-mouse CD44 mAb (IM7 mAb; BD Biosciences) or mouse anti-human CD44v3 mAb (CD44v3: R&D Systems), with horseradish peroxidase-conjugated rabbit anti-rat Ig (Dako) or 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

Rat anti-mouse CD44 (IM7) mAb recognises all CD44 isoforms (1-4)and mouse anti-human CD44v3 mAb recognises a CD44 isoform that carries HS side-chains (1-3). In mouse pancreas we have demonstrated that these two mAbs have a similar pattern of intra-islet staining.

References:

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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 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 4ºC.

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:
rat anti-mouse CD44 (IM7) mAb, BD Biosciences #553130 or
mouse anti-human CD44v3 mAb, R&D systems BBA11
horseradish peroxidase (HRP)-conjugated rabbit anti-rat Ig, Dako #PO450 (alternative HRP-rabbit anti-rat IgG, Sigma #A5795)
or HRP-rabbit anti-mouse IgG, Invitrogen #31450 (for use with CD44v3 mAb as primary ab)
Purified NA/LE Rat IgG_{2bk} , BD Biosciences #555845 or
Purified Mouse IgG_{2bk}, BD Biosciences#557351

6. Other reagents:
Hydrogen peroxide (30% w/w), Chem-Supply Pty Ltd (Australia) #HA154-500M,
Methanol, Merck # CAS-No. 67-56-1
3Amino-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.

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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% H_2O_2 + 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 Prepare citrate buffer, pH 6 for antigen retrieval. Dissolve 1.05 g Citric acid in 500 ml deionized waterand pH using 2 - 10 M NaOH.

7 Transfer slides to 250ml citrate buffer and heat in microwave (1600 watt) for 2 min on High power followed by 2 x 6 min on Low power.Allow the slides to cool on the bench for 30 min. Wash slides in 250 ml PBS, 3 x 10 min.

8 Wipe around sections using tissue. To block non-specific binding of Ig, apply animal free block (diluted to 20% v/v with deionized water) to tissue sections and incubate for 10 min at room temperature.

9 Tip off excess block in Step 8, wipe around sections using tissue and incubate with 40 µg/ml anti-CD44 (IM7) mAb (or 40 µg/ml rat IgG_{2bk} as isotype control; diluted in M.O.M. diluent), 125-150 µl/section at room temperature for 1 hour. Alternatively use 10 µg/ml mouse anti-human CD44v3 mAb (or 10 µg/ml mouse IgG_{2bk}as isotype control Ig.

Note: For human pancreas specimens, use 12.5 µg/ml mouse anti-human CD44v3 mAb or 12.5 µg/ml mouse IgG_{2bk} as isotype control

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

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11 Wipe around sections using tissue and incubate with 26-54 µg/ml secondary HRP-rabbit anti-rat Ig (Dako) or alternative 30-60 µg/ml HRP-rabbit anti-rat IgG (Sigma). For use with primary CD44v3 mAb, incubate with 8 µg/ml secondary HRP-rabbit anti-mouse IgG (Invitrogen).
Note: for human pancreas sections, use 6.4 µg/ml secondary HRP-rabbit anti-mouse IgG (invitrogen).

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

13 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.

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

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

16 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 (2x in 250 ml) and coverslip using glycergel mounting medium.

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

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