Integrated culture and purification of rat Schwann cells from freshly isolated adult tissue

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SUPPLEMENTARY METHODS

ISOLATION AND IDENTIFICATION OF FIBROBLASTS FROM ADULT RAT SCIATIC NERVE

Nerve fibroblasts were isolated from the outermost layer connective tissue of sciatic nerves (stripped off epineurium). The connective tissue was cut into small pieces and incubated in 0.05% (w/v) collagenase with agitation at 37 °C for 1 h. The cell suspension was filtered and centrifuged for 6 min at 400 g, room temperature. The supernatant was carefully removed and the remaining cell pellet was washed, re-suspended in fibroblast culture medium (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine and 100 U ml⁻¹ penicillin / 100 µg ml⁻¹ streptomycin) and plated in 25 cm² flasks. The cultures were characterized as fibroblasts by Thy-1 immunostaining. Fibroblasts were fixed with 4% (v/v) paraformaldehyde for 20 min, then washed three times with PBS (3x10 min) and incubated with 7.5% (w/v) bovine serum albumin (BSA) at room temperature for 1 hour followed by washing once with 1% BSA. Cells were then incubated with a phycoerythrin conjugated mouse anti-Thy-1 antibody (1:10) (AbD Serotec, UK) at room temperature for 45 min. Samples were washed three times with PBS (3 x 10 min) followed by nuclear staining with 300 nM 4’, 6-diamino-2-phenylindole (DAPI) (Sigma UK) for 10 min and washing with PBS (3 x 5 min). All images were taken using an epifluorescent microscope with xenon arc lamp source to excite phycoerythrin (λex = 480-565 nm / λem = 578nm) (Axon ImageExpress, Molecular Devices, USA). Phycoerythrin-mouse IgG1 isotype was used to determine the specificity of antibodies while Schwann cell cultures stained with Thy-1 antibodies were used as a negative control. Fibroblasts at passage 3-5 were used for experimentation.

CULTURE OF SCHWANN CELLS AND FIBROBLASTS FOR D-AMINO ACID OXIDASE (DAAO) OR GROWTH ANALYSIS

Fibroblasts were seeded at 1 x 10⁵ cells in 6-well plates (for RNA analysis) or at 8 x 10⁵ in 75 cm² culture flasks (for protein analysis) and cultured in either fibroblast culture medium or
Schwann cell culture medium. Cultures were maintained for 5 days (with a medium change at day 3) before total RNA or protein extraction for analysis of DAAO expression. Cultures were stained for Thy-1 or Schwann cell markers S100β, p75NGFR and GFAP (in separate well plates) to ensure that cultures contained fibroblasts and no contaminating Schwann cells before RNA or protein extraction. The expression of DAAO was determined in Schwann cells where cultures were maintained in Schwann cell culture medium for 5 days before RNA or protein analysis. To observe growth rate, fibroblasts (in either DMEM or Schwann cell culture medium) or Schwann cells were seeded at 1.8 x10^4 cells in 12-well plates and maintained for 18 days with medium a change every 3 days. The cultures were observed by light microscopy and imaged at days 3, 9 and 18.

**RT-PCR FOR D-AMINO ACID OXIDASE**

Total RNA was extracted from Schwann cell or fibroblast cultures by TRI Reagent® (Sigma, UK) and treated with RQ1 RNase-free DNaseI (Promega, UK) according manufacturer’s instructions. One microgram of total RNA from each sample was subjected to cDNA synthesis by random primers and ImProm-II™ reverse transcriptase (Promega, UK) according to the manufacturer’s instructions. Two microliters of synthesized cDNA was used for further PCR reactions for DAAO amplification. The separated reaction for alpha-actin amplification was used as a loading control. The reactions were held at 94 °C for 2 min before being subjected to 35 cycle amplification (30 cycles for alpha-actin) (denaturation at 94 °C, annealing at 55°C and extension at 72 °C) with final extension at 72 °C for 5 min. Forward primer 5´-AACACGAGCCTCCTTCTTG3´ and reverse primer 5´-TGGGAGAGCTGCTGTCAACT-3´ were used for DAAO amplification with a 398 bp product size. Forward primer 5´-CTGGCACCA CACCTTCTACA-3´ and reverse primer 5´-TGCCGATGGTGTGACCTG-3´ were used to amplify alpha-actin with a 500 bp product size. The PCR products were analyzed by agarose gel electrophoresis and genomic DNA was used as a positive control. Densitometry was conducted where the relative densitometry of the DAAO product was expressed as a function of the actin product.

**WESTERN BLOTTING OF CELL LYSATES FOR D-AMINO ACID OXIDASE**

Western blotting was conducted according to Kaewkhaw et.al. Ten micrograms of total protein from each sample and precision plus protein™ standards (Bio-Rad, UK) were separated on a 12% resolving polyacrylamide gel with a 5% stacking gel component (150 V, 2.30 hour) and transferred to a polyvinylidene fluoride (PVDF) membrane (300 mA, 1 hour). The membrane was blocked with 5% (w/v) nonfat-dry milk and incubated with goat anti-d-amino acid oxidase (DAAO; 1:250) (Everest Biotech, UK) overnight at 4 °C. Following washing, the membrane was incubated with an HRP-conjugated secondary anti-goat antibody (1:2000) (Everest Biotech, UK) for 1 h at room temperature. Membranes were then washed, incubated with an ECL
chemiluminescent substrate for 1 min and exposed to CL-Xposure Film. A kidney cell lysate was used as a positive control. Densitometry was conducted where the relative densitometry of the DAAO protein band was expressed as a function of the GAPDH protein band.

SCHWANN CELL CULTURE UNDER DIFFERENT MEDIUM CONDITIONS

Rat sciatic nerves were dissected bilaterally and Schwann cells were isolated as described in the protocol. Following cell harvesting and washing the cell pellet was re-suspended in 500 µl of DMEM containing d-valine (DMEM-d valine) supplemented with 10 % (v/v) fetal bovine serum. A cell suspension of 60 µl was seeded in 12-well plates containing different conditions of culture medium. Six sample groups were tested to determine an importance of mitogenic factors present in medium in supporting Schwann cell growth. All mitogens (forskolin, bovine pituitary extract and N2 supplement) were withdrawn from the complete Schwann cell culture medium (DMEM-d valine plus supplements, see in protocol) and used for cell culture. Cells were also tested with DMEM-d valine containing only forskolin. Cultures in complete Schwann cell culture medium were used as a positive control while cells in DMEM-d valine were used as a negative control. Samples were maintained under different medium conditions for 20 days (with medium changed every three days). Samples from each group were immunolabelled for S100β protein and counterstained with DAPI. All images were taken using an epifluorescent microscope. Purity was determined as described in the protocol, while Schwann cell density was derived from the number of Schwann cells (S100β-positively stained cells) per square centimeter (each micrograph field of view = 0.013 cm²).

REFERENCES