Susceptibility to oxidative stress-driven injury of respiratory epithelium from children with Down syndrome

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Online Supplement

Material and Methods

Sampling and culturing of primary nasal epithelial cells

Primary nasal epithelial cells (PNECs) were obtained from children with DS who visited the outpatient clinic or day-care center of the Emma Children's Hospital/Academic Medical Center, Amsterdam, the Netherlands, for non-pulmonary disorders. Children with a normal karyogram served as controls. Children with concurrent respiratory infections were excluded.

A cytology brush (Cytobrush Plus, CooperSurgical, Trumbull, CT, USA) was introduced 1-2 cm into the nose and rotated along the nasal cavity. The cells were washed in a solution of 50% PBS and 50% RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) and cultured at 37°C in ambient air, supplemented with 5% CO_2 in collagen-coated (Vitrogen-100, Cohesion Technologies, Palo Alta, CA, USA) cell culture plates (Corning Costar, Schiphol-Rijk, The Netherlands) in BEGM-Bulletkit bronchial epithelial medium containing the following supplements/growth factors: bovine pituitary extract, hydrocortisone, hEGF, epinephrine, transferrin, insulin, retinoic acid, triodothyronine, and gentamycin/amphotericin-B (Cambrex Cooperation, East Rutherford, NJ, USA) and ciproxin (Bayer BV, Mijdrecht, The Netherlands). After ~2 wks of culturing and 1-2 passages, the PNECs were used for experiments at ~90% confluency.

Determination of PNEC purity

A cytospin preparation of a PNEC culture was fixed in 4% paraformaldehyde, treated with peroxidase block (Envision system, DAKO, Carpinteria, CA) and blocked with 3% bovine serum albumin in PBS/TritonX-100 0.1%. Thereafter, the cells were exposed to a pancytokeratine antibody containing the following primary antibodies: mouse anti-human cytokeratin 1-8, 10, 13-16 and 19 mAb (AE1/AE3 clone, DAKO), for 1 hour at RT, followed by incubation with labeled polymer-HRP anti-mouse and AEC+ substrate chromogen (Envision system, DAKO) according to the manufacture's description. The cells were counterstained with haematoxylin.

Intracellular oxidative stress

To demonstrate the production of intracellular oxidative stress, cells were incubated with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCFDA, Invitrogen, Carlsbad, CA, USA) for 30 minutes in fenolred free Dulbecco's Modified Eagle Medium (Invitrogen). DCFDA's fluorescent signal upon exposure to oxidative stress was measured with a plate reader (Fluostar Optima, BMG Labtech, Ortenberg, Germany) and FACSCalibur flow cytometer (BD, Franklin Lakes, NJ, USA).

Protein expression

SOD1, catalase and GPX expression were measured by separation on SDS-PAGE followed by Western blotting. Variation in loading was normalized by correlation to actin or tubulin-content. Primary antibodies were rabbit-anti-human SOD-1, diluted 1:1000 (HPA001401, Sigma-Aldrich, St. Louis, MO, USA), goat-anti-human actin, diluted 1:1000 (sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit-anti-human GPX-1/2, diluted 1:100 (sc-30147, Santa Cruz Biotechnology), goat-anti-human catalase, diluted 1:200 (sc-34281, Santa

Cruz Biotechnology) and mouse-anti-human tubulin, diluted 1:5000 (T6199, Sigma-Aldrich). Bound antibodies were visualized by IRDye conjugated donkey-anti-rabbit/goat/mouse polyclonal antibodies, diluted 1:15.000 (926-68023 /926-32214/926-32212/926-32222, LI-COR Biosciences, Lincoln, NE, USA). Quantification was performed by infrared fluorescence detection using the Odyssey Imager and software (LI-COR Biosciences).

mRNA measurement

After exposure to CCCP for 24 hours, Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used according to the manufactures description to lysate the cells and isolate mRNA. The mRNA was then stored at -80°C. mRNA content was measured by Multiplex ligation-dependent amplification (MLPA), using a mRNA apoptosis probe mix (MRC-Holland, Amsterdam, The Netherlands, figure E1-2 of the online supplement).

At the time of mRNA analysis, we were aware that FACS analysis had showed that the percentage of apoptotic PNECS after exposure to oxidative stress in DS was not different from controls, which made differences in apoptosis regulation unlikely. Therefore, as a first step, we pooled mRNA samples of 4 patients with DS and 4 controls and planned to pursue analysis per individual sample in case the pooled samples suggested differences between DS and controls. This was not the case and thus the second step was not performed.

Figure Legend Online Supplement

Figure E1. mRNA content as measured by Multiplex ligation-dependent amplification (MLPA) in primary nasal epithelial cells (PNECs) from control children (n=4) after exposure to 0-6-25-100 uM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) for 24 hrs, using a mRNA apoptosis probe mix. (Down syndrome: n=4; controls: n=4).

Figure E2. mRNA content as measured by MLPA in PNECs from children with Down syndrome (n=4) after exposure to 0-6-25-100 uM CCCP for 24 hrs, using a mRNA apoptosis probe mix.