Inhaled corticosteroids reduce senescence in endothelial progenitor cells from COPD patients

ONLINE DATA SUPPLEMENT

Material and Methods

Participants

Blood samples (15–48 mL) were collected from healthy non-smoking volunteers, smokers with normal lung function (forced vital capacity in 1 second (FEV_1) >80% predicted, FEV_1/forced vital capacity (FVC) >0.7) and COPD patients (FEV_1<80% predicted, FEV_1/FVC <0.7). All individuals aged 38 to 80 years, and were free from significant cardiac, renal, haematological, or other major disorders as determined by medical history, physical examination and screening investigations. All COPD patients were current or ex-smokers and were classified according to the Global initiative for chronic Obstructive Lung Disease (GOLD) criteria for severity of disease. All volunteers were stable (no chest or other infection requiring antibiotics and/or oral steroids) for at least 4 weeks. The study was approved by the Royal Marsden, Hammersmith and Queen Charlotte's and South East Scotland Ethics Committees, and informed consent was obtained from all individuals.

Isolation and Culture of ECFC from Peripheral Blood

Peripheral blood mononuclear cells were isolated from blood samples and seeded at a density of 3–5 × 10^7 cells per well, in complete endothelial growth medium (EGM)−2 (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Thermo Scientific, Fisher Scientific Ltd., Loughborough, U.K.), onto six-well plates precoated with type I rat tail collagen (BD Biosciences, Bedford, MA), as previously described[1]. After 24 hours, nonadherent cells and debris were aspirated, adherent cells were washed once with EGM-2 medium, and fresh EGM-2 was added to each well. Medium was changed daily for 7 days and then every 2 days. Colonies of ECFC appeared between 7 and 22 days in culture as discrete colonies of cells with cobblestone morphology and were enumerated by visual inspection using a ×4 objective lens (EVOS™ XL Core Imaging System). Endothelial cells derived from the colonies were passaged for 2–3 weeks after appearance and grown to confluence. All experiments were performed with ECFC between passage 3 and 5. There were no differences in the isolation success rate between the groups as described in supplemental material of reference.[1]

Stress Induced Premature Senescence by Oxidative Stress

We used a previously published method for inducing premature senescence by H_2O_2 (ref Stem Cells). Commercially available human umbilical vein endothelial cells (HUVEC) from pooled donors were used for our experiments (Lonza). HUVEC 1×10^5 were seeded in six-well plates and grown to 80% confluence in M199 medium (Sigma-Aldrich Company Ltd., Dorset, U.K.) plus 10% FBS. Following 1 hour pre-treatment with budesonide or control vehicle (DMSO), cells were washed twice with PBS and treated for 1.5 hours with 50 µmol/L of H_2O_2 (Sigma-Aldrich Company Ltd.). Cells were washed twice with PBS and cultured in M199 plus 10% FBS medium for three additional days, in the absence or presence of increasing doses of budesonide (Sigma-Aldrich Company Ltd) between the range 10^{-6}M and 10^{-10}M.

Immunofluorescence

Isolated ECFC or HUVEC were stained as previously described[1] with antibodies to vascular endothelial (VE)-cadherin (CD144) (AF938, Bio-Techne Ltd, Abingdon, Oxfordshire, U.K.), p16 and p21 (sc-65224 and sc-817 Santa Cruz Biotechnology, Insight Biotechnology Ltd., Wembley, U.K.), 53 binding protein 1 (53BP1) (4937, Cell Signaling Technology, New England Biolabs, Hertfordshire, U.K), γ-H2AX (05-636, Millipore) and IP-10 (MA5-32674, Invitrogen Ltd). Secondary antibodies were anti-mouse AlexaFluor 647, anti-rabbit AlexaFluor 488, and anti-goat Alexa Fluor 555 (Invitrogen Ltd). Nuclei were visualized using deep red anthraquinone 5 (DRAQ5) (Biostatus Limited) or 4-6-diaminidino-2-phenylindole (DAPI) (Thermo Fisher Scientific).

Senescence-Associated β-Galactosidase Staining

Senescence-associated β-galactosidase (SA-β-Gal) activity was measured with a β-Galactosidase staining kit (Senescence Detection Kit, BioVision Research Products, Mountain View, CA) following the manufacturer's protocol. The number of blue (senescent) cells relative to the total cell number was counted in two to four different optic fields, using ×10 or ×20 objective lens. At least 200 cells were counted per sample.

Caspase-Glo 3/7 Assay

Apoptosis was quantified by measuring caspase 3 and 7 activation, using Caspase-Glo 3/7 Assay (Promega, Southampton, United Kingdom) on a Bio-Tek Synergy HT multidetection microplate reader, following the manufacturer's protocol.

Western Blotting

Western blotting was carried out as described[1]. The following antibodies were used: p21 Waf1/Cip1 (Cell Signaling Technology, 2947), γ-H2AX (ser139) (Cell Signaling Technology, 9718), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Millipore, Watford, U.K.), α-

tubulin (Sigma-Aldrich Company Ltd.). Quantification of protein levels was performed by densitometry and normalized against GAPDH or α-tubulin.

Real-Time Polymerase Chain Reaction

RNA was extracted from ECFC or HUVEC using the RNeasy kit (Qiagen), according to the manufacturer's instructions. After reverse transcription (QuantaBio qSCRIPT cDNA Supermix, VWR Cat No. 733-1177), p21 mRNA levels were measured by quantitative real-time polymerase chain reaction (RT-PCR) using 5ng cDNA per test well and SYBR Green technology (Biorad iQ SYBR Green Supermix, Cat No. 1708882). Levels of p21 were normalized to Ribosomal Protein L13a (RPL13A), which is an appropriate housekeeping gene for studies on senescence for ECFC[2]. All measurements were performed in triplicate. Primer sequences are as follows: p21 forward: 5'-GCAGACCAGCATGACAGATTT-3', reverse: 5'-GGATTAGGGCTTCCTCTTGGA-3'; RPL13A forward: 5'-CTGGACCGTCTCAAGGTGTT-3', reverse: 5'-GCCCCAGATAGGCAAACTT-3').

Luminex assay

Human cytokines were measured in ECFC supernatant (5 non-smokers; 8 COPD patients, 3 no-ICS and 5 on ICS). 100,000 cells were seeded in 6 well-plates in 1.3 ml of normal medium (EGM2, baseline conditions). Supernatant was collected after 48 hours.

Thirty-two human cytokines were assayed using the Luminex MAGPIX Analyzer (Austin, TX, U.S.A.) as previously described[3]. The mean fluorescent intensity was analysed using a five-parameter logistic method on XLfit software v.5.3.1.3 (Guildford, Surrey, U.K.). Twenty-two analytes were detected (please see table below: undetected analytes are displayed in grey).

GM-CSF	IL-17A	**IL-6**	**RANTES**
G-CSF	**IL-1rα**	**IL-7**	TNF-α
IFN-α-2	**IL-1α**	**IL-8**	**TNF-β**
IFN-γ	IL-1β	**IP-10**	**Eotaxin**
IL-10	IL-2	**MCP-1**	**VEGF**
IL-12p40	IL-3	**MCP-3**	EGF
IL-12p70	**IL-4**	**MIP-1α**	**Fractalkine**
IL-13	IL-5	**MIP-1β**	**GRO**

Imaging and Image analysis

At least 5 representative image stacks for quantification were captured for each sample/individual on a Zeiss LSM-780 inverted confocal laser scanning microscope using either a ×40, or x63 oil objective. The images were analysed using FIJI image analysis software and macros were developed to quantify either the DNA damage foci per nucleus, using the DAPI or DRAQ5 fluorescent signal to create masks, or to measure the nuclear and cytoplasmic intensities for each sample.

Statistical Analysis

Data are expressed as mean ± SEM or ± SD as described. Statistical analysis was performed with GraphPad-Prism 9. Comparisons were performed with Mann-Whitney U test, Kruskal Wallis test followed by Dunn's post-hoc analysis (for unpaired samples) or Friedman's test followed by Dunn's post-hoc analysis (for paired samples - experiments on HUVEC). The correlation of values was estimated with the Pearson *r* correlation coefficient. Significance was defined as $p<0.05$.

References

We would like to include the following references some of which we were unable to include in the main manuscript due to number limitations

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