Online Supplement

Title: MMP-12 SNP affects MMP activity, airway macrophage infiltration and emphysema in COPD

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Cell Culture and Vector Transfection

COS7 cells were cultured in a humidified atmosphere with 10% CO_2 at 37°C with Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum and 2mM L glutamine. Passages occurred every 5 – 7 days using 1x Trypsin/EDTA

Cells were plated in six well plates in 3 ml medium. Cells were cultured until 90 – 95% confluence before transfection. Transfection of MMP-12 containing vectors (250ng) was carried out using Fugene 6 (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Analysis of enzymatic activity

MMP-12 constructs and Null pCMV – XL4 (no insert) vector were transiently transfected into COS7 cells. After 24 hours incubation, the medium was replaced with serum free medium before conditioned media was collected in the next 24 time period. The conditioned media was stored at – 80^{o}C until assayed.

Initial analysis of MMP-12 enzymatic activity in supernatants was determined by casein zymography. Zymography was performed as follows; precast sodium dodecyl sulfate - polyacrylamide gels (7.5%), containing 12% casein (Invitrogen, Paisley, UK), were used to separate samples in 2 × nonreducing sample buffer (0.125M Tris-HCI, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate, 0.003% bromphenol blue) at 120V. Sodium dodecyl sulfate was

removed with reanatuing buffer (2.5% Triton X-100) (Invitrogen, Paisley, UK) for 30, minutes at room temperature. The gels were incubated overnight hours at 37°C (Heraeus Incubator, Langenselbold, Germany) in developing buffer (20mM Tris-HCl, pH 7.6, 10mM $CaCl_2$ and 0.04% NaN_3) (Invitrogen, Paisley, UK) and then stained with 0.1% Coomassie blue in 40% methanol and 10% acetic acid and destained until clear proteolytic bands appeared on the contrasting blue background. Bands were visualized where MMP-12 had degraded the casein matrix, leaving a clear band after staining the gel for protein, differential activity or amounts of MMP-12 creates different band intensities. Every gel included as a standard 10 ng of recombinant human MMP-12 (R&D Systems, Minneapolis, USA).

The activity of MMP-12 relative to the standard was determined by densitometry of the bands using ImageJ a Java-based image processing program by the National Institutes of Health. The intensity and width of the bands were taken into consideration.

To provide a specific read out of activities the commercially available Sensolyte 520 MMP-12 Assay Kit (Anaspec, San Jose, CA) was used. It detects MMP-12 activity using a 5-FAM/QXL™520 fluorescence resonance energy transfer (FRET) peptide. In the intact FRET peptide, the fluorescence of 5-FAM is quenched by QXL™520. Upon cleavage into two separate fragments by MMP-12, the fluorescence can be monitored at excitation/emission wavelengths = 490 nm/520 nm. Detection was performed using a Flexstation with Softmax Pro software (Molecular Devices, Sunnyvale, CA). Assays were performed in triplicate according to company protocol.

Cell Viability

To monitor cell viabilty between transfections the MTT assay was used. The MTT assay is based on the ability of mitochondrial dehydrogenase enzyme from viable cells to cleave the tertrazolium rings of pale yellow MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide] and forms blue formazan crystals which are accumulated within healthy cells. The number of viable cells is directly proportional to the level of formazan (24).

Transfected cells had the media removed and had MTT containing phenol red free media, prewarmed at 37^{o}C, added at a concentration of 0.5 mg/ml. This was then incubated for 2 – 4 hours.

At the end of the incubation period the medium was removed and the formazan crystals were solubilised with 250µl of DMSO. The sample was then transferred to a 96 – well microtitre plate. Colormetric measurements were made using plate reader (ThermoScientific Multiskan Ex, Waltham, Massachusetts, USA) set at a wavelength of 650 nm, reads for each sample were performed in triplicate, and mean values calculated.

Total Protein Levels

The assay is based on absorbance maximum change for Coomassie Brilliant Blue G-250 (Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories, Hemel Hempstead, U.K) which shifts from 465nm to 595nm when protein binding occurs.

From each sample to be measured 10µl of the supernatants and taken were added to 500µl of 1:5 diluted Protein Assay Dye Reagent Concentrate with sterile distilled H_2O. The samples were then incubated for 5 minutes before 200µl were added to two wells of a microtitre plate, providing a duplicate reading from which a mean value was calculated. The optical density was read at 595nm before 1 hour had passed using a plate reader (ThermoScientific Multiskan Ex, Waltham, Massachusetts, USA).

MMP-12 Expression Levels

To produce RNA, the medium was aspirated and washed with PBS and trypsinised as for passage. An RNeasy mini kit (Qiagen, Crawley, U.K) was then used according to manufacturer's instructions.

Superscript II first strand synthesis system for RT-PCR (Invitrogen, Paisley, UK) was used, to produce cDNA from the RNA. All temperature conditions were performed using a DNA Engine Tetrad™ Cycler system (MJ Research, Massachusetts, USA).

A master mix containing 5µl total RNA, 1µl (100ng) random hexamers, 1µl 10mM dNTPs and 3µl H_2O per reaction is used. This is then incubated at 65^oC for 5 minutes before placing on ice then room temperature.

Another mastermix is then added consisting of 2µl 2X RT buffer, 4µl 25mM $MgCl_2$, 2µl 0.1M dithiothreitol and 1µl (40U) RnaseOut. Superscript II Reverse transcriptase (200U) of volume 1µl was also added to RT + samples, whilst 1µl H_2O was added to RT – controls. Samples were then cycled as follows; 25^oC for 10 minutes, 42^oC for 50 minutes and 70^oC for 15 minutes. Samples were then stored at -20^oC until use in real time PCR reactions.

The Brilliant SYBR® Green QPCR Master Mix system (Agilent Technologies, California, USA) with a Stratagene Mx3005 QPCR light cycler (California, USA) was used to perform real time PCR.

Primers were to the exonic sequence of MMP – 12 and β – Actin which was selected as an internal control. Primers used are as follows (Invitrogen, Paisley, UK):MMP – 12 Forward: 5' – CATTCAGGAGGCACCAACTTGTTC – 3' MMP – 12 Reverse: 5' – CCTTTGGATCACTAGAATGGCC – 3' β – Actin Forward: 5' – GGATGCAGAAGGAGATTACTG – 3' β – Actin Reverse: 5' – CGATCCACACAGAGTACTTG – 3'

To perform the real time reaction, each set of forward and reverse primers were mixed to give a 5µM stock. Also a 25 x dilution of cDNA synthesis reaction was made. Every cDNA sample was run in triplicate for each set of primers.

For each sample, the following mastermix is made; 12.5µl of Brilliant SYBR® Green QPCR Master Mix, 5µl of diluted cDNA, 6.5µl of diluted cDNA and 1µl of 5µM primer mix.

The samples were then placed in the light cycler, which was set to read SYBR green and set to report fluorescence during both the annealing and extension step of each cycle. The following PCR program was then used 1 cycle at 95^{o}C for 10 minutes, and 40 cycles of 95^{o}C for 30 seconds, 55^{o}C for 1 minute and 72^{o}C for 1 minute.

Triplicate values were averaged and the difference between the ΔCT value for housekeeping primers, and the ΔCT value for the gene was calculated to give the ΔΔCT value.

Minigene Analysis of Splicing

Pre-mRNA splicing is when intron sequences are removed and exons are aligned to generate a mature mRNA transcript (25). Spliceosomes, are key to this process. They recognise boundaries through short regulatory motifs of 6-8 nucleotides in both introns and exons which can enhance or silence splicing. Disruption of an enhancer or silencer by polymorphism could result in significantly altered gene function. The effect of the rs652438 polymorphism on possible exonic splice silencing was analysed using the minigene technique.

MMP-12 exon 8 and the flanking intronic sequence representing A and G alleles of rs652438 were amplified from genomic DNA of homozygotic individuals from the EU COPD cohort. The primers used were: forward 5' – tcccatgtcgacCCATGGGAACCATAGAAAAGA – 3 and reverse 5' -gcagccgcggccgcTCAGAAACCAAAAACACAAAGAA – 3'. The primers were also designed to introduce Sal I and Not I restriction sites (underlined) in the 5' and 3' ends of

the prospective insert. Lowercase indicates genomic DNA sequence complimentary anchors attached to primers to improve annealing.

DNA was amplified in reaction mixtures containing 200 µM each of dATP, dCTP, dGTP and dTTP (Fermentas Life Sciences, York, U.K), in a 1x (dilution of 10x) commercial buffer containing; 50mM KCl 10mM tris-HCl pH 8.3 and 1.5mM $MgCl_2$, 50 mM $MgCl_2$, 1 U *Taq polymerase* (Bioline, London, U.K) on a DNA Engine Tetrad™ Cycler system (MJ Research, Massachusetts, USA).

The conditions were 94°C for 40 s, and 35 cycles of denaturing at 94°C for 30s, primer annealing at 60°C for 1min, and extension at 72°C for 1 min, with a final extension of 10 min at 72°C. PCR products were visualized by electrophoresis on 1% agarose gels stained with ethidium bromide.

The PCR fragment was 525 bp (166 bp of intron 7, 160 bp of exon 8, and 199 bp of intron 8). This was cloned into TOPO.2.1 vector according to manufacturer's instructions (Invitrogen, Paisley, U.K).

Inserts were sequenced after DNA mini-preparation (Qiagen, Crawley, U.K). Reactions were carried using topo vector forward and reverse primers. 30–50ng of PCR product and 5 p – moles of the primer were used for ABI Big Dye terminator cycle sequencing (Applied Biosystems, Foster City, USA). For the sequencing reaction 2.5µl of purified PCR product was used in a final reaction volume of 10µl. Sequencing was performed as described by the protocol. The products were then outsourced for the gel sequencing reaction. Sequencing alignment was done using EBI-tools ClustalW web-based software (http://www.ebi.ac.uk/Tools/clustalw2/index.html) to confirm that the inserts were identical apart from the base at the SNP.

Restriction digestion of the TOPO TA constructs and exontrap vector (MoBiTec, Göttingen, Germany) using SalI and NotI in 10µl double digest reactions (Promega, Southampton, U.K). 5 units each was used, 1x digestion buffer 2 µg DNA for 4 hours at 37°C. Followed by inactivation step of 65°C for 10 min.

Digested vectors had gel electrophoresis performed on a 1% gel at 120v before gel extraction (Qiagen, Crawley, U.K). Vector and insert (1:3 ratio) were ligated using T4 DNA ligase and 1x Ligation buffer (Promega, Southampton, U.K) in 10 µl reaction at 4°C overnight. Vectors were transformed into X1 –blue supercompetent cells carried out according to the manufacturer's instructions (Stratagene, California, USA).

Screened colonies were had DNA mini prep (Qiagen, Crawley, U.K) performed on then sequenced with exontrap vector primers. Successfully cloned exontrap vectors were bulked up for mammalian transfection and plasmid purified using an endotoxin-free midi kit (Qiagen, Crawley, U.K).

COS 7 cells were then transfected with exontrap vectors before having RNA extracted and cDNA synthesis performed as described previously.

The amplification of cDNA was carried out in 10 µl reactions, containing 1 µL of cDNA, 1XTaq buffer with $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 mM of each vector primer, and 1 unit of Taq polymerase. The conditions for amplification were as follows: 94^0C for 3 minutes, 20 cycle touchdown of 65^0C to 55^0C for 30 seconds per cycle, 20 cycles of 94^0C for 30 seconds, 55^0C for 30 seconds, 72^0C for 60 seconds and a final extension at 72^0C for 5 minutes.

Migration Assay Investigation of MMP-12 Variants

Transwells, 6.5 mm in size with 8µm pores (Fisher Scientific, Loughborough, U.K) were coated with collagen IV (Sigma, Gillingham, U.K) at 0.1 mg/ml in serum free media with 0.1% bovine serum albumin (BSA) added. A total of 700µl collagen in media is required per well with 100µl for the top well and 600µl for the bottom. This was then incubated at 4^{o}C for 24 hours.

After 24 hours the collagen media was aspirated and replaced with serum free media containing 0.1% BSA in the same manner as the collagen containing media was added previously. Incubation was for 4 hours at 37^{o}C before aspiration.

COS7 cells were transiently transfected with rs652438 constructs before replacing of the media 24 hours after transfection and adding serum free media to perform serum starvation 24 hours prior to the experiment. Cells was then trypsinised as if for passage and resuspended in a small amount of serum free media containing 0.1% BSA.

To ensure a comparable experiment cells were diluted to a density of 8 x 10^{5}/ml and 100µl cells added to the top well. The bottom wells were filled with 600µl 10% fetal calf serum containing media which acted as a chemotactic factor. The transwells were then incubated for 8 hours at 37^{o}C and 5% CO_2 in a Hereus incubator (Thermo-Electron Corporation, Waltham, Massachusetts, USA).

To stop the experiment, media was aspirated from both the bottom and upper well before cells were fixed by replacing with 4% formaldehyde. This was then kept until ready for staining and imaging. After aspirating formaldehyde, remaining cells in the upper chamber were removed using a cotton bud. DAPI at 1µg/ml for 30seconds was then used to stain the cells before washing in H_2O.

Transwells were then viewed using a wide field fluorescence microscope (Diaphot 400, Nikon, Tokyo, Japan) at 100 x magnification. Six random pictures of each well were taken

using Insight QE camera (Mikron instruments, San Marcos, California, USA), all experiments were blinded and performed to n = 3.

Investigation of Inflammatory Cell Numbers

Inflammatory cell migration was investigated in a small initial cohort detailed by Lowrey. The Nottingham Research Ethics Committee approved the collection of this resource. Smokers with COPD defined by GOLD criteria and smokers without COPD were recruited. Patients were aged 40–80 years, had $\geq$ 10-pack year smoking history and had not required antibiotics or oral steroids for the previous 6 weeks. Patients with α1-antitrypsin deficiency, radiological evidence of interstitial lung disease, previous thoracic surgery, or taking inhaled corticosteroids were excluded. Spirometry was performed pre and post bronchodilator according to American Thoracic Society guidelines and breathlessness was recorded using the Medical Research Council (MRC) dyspnoea score. Those with reversibility greater than 10% of baseline FEV_1 after inhaling 400 mcg of salbutamol were excluded. Subjects with FEV_1/FVC of less than 70% were classified as having COPD.

Differential cell counts were performed according to standard methods. Genotypes were determined sequencing of the rs652438 SNP. Collection of cohort and differential cell counts were performed by Gill E Lowrey.

Replication was performed in the ECLIPSE cohort. A total of 2,180 COPD patients aged 40–75 yrs, with baseline post-bronchodilator FEV_1 of <80% of the predicted value, baseline post-bronchodilator FEV_1/forced vital capacity (FVC) of ≤0.7 and a smoking history of ≥10 pack-yrs were recruited. Exclusion criteria included respiratory disorders other than COPD, prior medical history of significant inflammatory disease other than COPD, COPD exacerbation 4 weeks prior to recruitment, lung surgery, diagnosis of cancer, blood transfusion 4 weeks prior to recruitment, inability to walk, taking part in blinded drug studies, therapy with oral

corticosteroids and participation in radiation exposure studies. Information on a number of COPD specific phenotypic endpoints was obtained. For the interest of replicating findings in the Lowrey cohort, absolute cell number counts of inflammatory cells were obtained for those individuals where induced sputum was taken. The Illumina 550 genome wide platform was used to provide genotype information for rs652438 for individuals in the ECLIPSE cohort.

Investigation of Emphysema Scores

SNP investigation with regards to emphysema score was also performed in ECLIPSE. Emphysema scores were obtained using computerised tomography (CT) performed at full suspended inspiration. A multi – slice CT scanner was used, with contiguous images taken at 1 or 1.25 mm slice thickness. Images were obtained at 120kVp and 40mAs. Image analysis was performed using a low spatial frequency reconstruction algorithm (GE-standard, Siemens – b35f).