PMN degranulation in relation to CD63 expression and genetic polymorphisms in healthy individuals and COPD patients

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Abstract. Polymorphonuclear neutrophils (PMNs) play an important role in chronic obstructive pulmonary disease (COPD) pathogenesis. The tetraspanin CD63 is a membrane marker of azurophilic granules and is actively involved in the process of PMN endocytosis and azurophilic granule exocytosis. In this study, we investigated genetic polymorphisms of the *CD63* gene, quantified CD63 expression and PMN myeloperoxidase (MPO) release in healthy individuals and COPD patients. We evaluated the potential correlations between genetic polymorphisms and gene expression and MPO release. COPD patients had significantly lower CD63 expression and released less MPO upon chemokine stimulation compared with the healthy individuals. Eleven putative polymorphisms in the *CD63* gene were investigated but only three were polymorphic in our study subjects. None of the polymorphisms was associated with *CD63* expression in either the healthy subjects or the COPD patients. However, the 8041C/G polymorphism, which is located 3' to the *CD63* gene, was associated with MPO release in the healthy subjects. The CC genotype was associated with greater MPO release than the GG genotype (P=0.007). These results suggest that COPD patients have different patterns of *CD63* expression and PMN mediator release than healthy individuals. It is likely that genetic variants have limited effect on *CD63* expression and MPO release in the context of COPD but their role in other diseases has yet to be determined.

Introduction

Chronic obstructive pulmonary disease (COPD) is one of the most common pulmonary diseases worldwide. The major underlying pathophysiological processes are proteolytic destruction of connective tissue of the lung and inflammatory narrowing of peripheral airways (1). Cigarette smoking is clearly the major environmental risk factor for COPD development. However, only 10-15% of smokers develop severe impairment of pulmonary function associated with COPD (2). Family and twin studies have documented familial aggregation of lung function even after adjustment for cigarette smoking (3,4). A few genetic risk factors have been identified in linkage and association studies (5) and taken together these data indicate that COPD is a complex heterogeneous disease with multiple genetic factors involved in its pathogenesis.

Polymorphonuclear neutrophils (PMNs) are one of the major effector cells in the process of chronic airway inflammation in COPD (6). Increased numbers of PMNs have been found in induced sputum, in bronchial biopsy specimens, and in bronchoalveolar lavage fluid from COPD patients (7,8), and this increase was associated with the severity of airflow limitation (9) and the rate of decline of lung function (10). PMNs synthesize a large number of proinflammatory cytokines/chemokines, proteinases, lipid mediators, and store them in various granules. The proteinases stored in azurophilic granules, are capable of degrading almost all the components of extracellular matrix, and they are closely associated with tissue damage and the development of inflammation (11,12). Unrestrained proteinase activity in general and elastase specifically are currently believed to play a crucial role in the pathogenesis of emphysema, one of the major pathologic features of COPD (13).

The mechanisms underlying azurophilic granule mobilization and release are still poorly understood. CD63 is an azurophilic granule membrane protein that belongs to the tetraspanin protein superfamily, which consists of at least 32 widely expressed members in mammals (14). Tetraspanins regulate many important cellular functions, such as migration, fusion and signaling events by interacting with a diverse array of molecules (15). Translocation of CD63 to the plasma membrane is associated with PMN stimulation and azurophilic granule exocytosis (16). Therefore, upregulation of CD63 on the plasma membrane surface of PMNs has been considered as an excellent marker for azurophilic granule release (17). However, the precise biological functions of CD63 in the process of azurophilic granule mobilization and mediator release have yet to be fully established. Studies performed in other cell types have suggested that CD63 is involved in cell activation and mediator release. For example, in a basophilic leukemia cell line, anti-CD63 antibody stimulated cells to secrete high levels of granule contents (18). In eosinophils,

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translocation of CD63 is associated with selective mediator release from crystalloid granules (19).

Since there is a genetic component to COPD pathogenesis, and PMN proteinases play an important role in COPD development we hypothesized that polymorphisms could affect *CD63* expression, alter PMN activity, and modulate the susceptibility to COPD. Herein, we characterized genetic polymorphisms of the *CD63* gene, measured its mRNA and protein levels in PMNs, quantified myeloperoxidase (MPO) release upon interleukin (IL)-8 stimulation, and evaluated the potential correlation of the genetic polymorphisms with *CD63* expression and PMN degranulation activity.

Materials and methods

Study subjects for polymorphism discovery and gene expression analysis. The gene expression study was performed in two groups of subjects: healthy individuals and COPD patients. This was because the expression pattern of *CD63* and its genetic regulation could be influenced by differences in the cellular milieu of the PMNs due to the disease process. A total of 60 Caucasian healthy volunteers (33 female/27 male) and 70 Caucasian physician-diagnosed COPD patients (29 female/41 male) were recruited for the functional study (Table I). The healthy volunteers were mainly colleagues working in the authors' laboratory. The COPD patients were outpatients from St. Paul's Hospital and Vancouver General Hospital. The disease diagnosis was based on the GOLD criteria, that is, significant exposure to cigarette smoke, and not fully reversible airflow limitation (forced expiratory volume in 1 s/forced vital capacity <0.7) (20). The mean ages of the control and patient groups were 34.7±10.4 years and 69.8±10.6 years, respectively. All the participants gave written informed consent and donated 20-40 ml of peripheral blood. The experimental protocol for this study was approved by the Providence Health Care Research Ethics Board.

Table I. Characteristics of the study subjects (continuous variables are shown as mean ± SD).

Characteristic	Healthy subjects	COPD patients
Ethnicity	Caucasian	Caucasian
Number of subjects	60	70
Age (Years)	34.7±10.4	69.8±10.6
Gender (F/M)	33/27	29/41
FEV1 (% of predicted)	Not done	48.5±17.1
FEV1/FVC (%)	Not done	50.2±28.5
Former/current smoker (n)	13	68
Smoking (pack-years)	1.9±4.7	47.0±31.4
Medication history		
Oral corticosteroid	0	8
Inhaled corticosteroid	0	57
Short acting ß$_2$-adrenergic receptor agonist	0	36
Long-acting ß$_2$-adrenergic receptor agonist	0	19
Theophylline	0	9

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity.

PMN isolation and stimulation. PMNs were isolated by a Dextran-Ficoll sedimentation and centrifugation method (21). The contaminating erythrocytes were removed by hypotonic lysis. The isolated PMNs were subject to Kimura staining and microscopic cell differential count and the purity of the PMNs was calculated. The preparations usually contained >98% neutrophils. Samples with >2% eosinophils were excluded from the study. Isolated PMNs were incubated on ice for 40 min before proceeding to further experiments.

Measurement of MPO release. An aliquot of 5-10x10^6 cells/ml in phenol red-free RPMI-1640 was primed by 10 nM fMLP (f-Met-Leu-Phe) at 37˚C for 2 min followed by 100 ng/ml IL-8 stimulation at 37˚C for 10 min. Stimulated PMNs and an identical aliquot of unstimulated PMNs were centrifuged at 2500 rpm for 3 min. The supernatant and cell pellets were kept at -70˚C for the mediator release assay. The unstimulated cell pellet was homogenized by short sonication, and then lysed by electroporation using 3 discharges of 6.25 kV/cm, 25 μF, and 50 Ω using Gene Pulser II (Bio-Rad, Hercules, CA).

MPO release was measured as previously described (22,23). Briefly, 100 μl of the sample was mixed with 150 μl tetramethylbenzidine substrate in a 96-well microplate and incubated at room temperature for 15 min. The reaction was terminated by addition of 50 μl 1 M sulfuric acid. The absorbance was read at 450 nm in a spectrophotometric microplate reader (Spectra & Rainbow ELISA reader: Tecan, Zurich, Switzerland). The amount of released MPO was calculated as a percentage of total cellular MPO by dividing the absorbance of the supernatant from the stimulated cells by the sum of absorbance from the supernatant and lysed cell pellet under resting conditions (24).

RNA extraction and cDNA synthesis. PMNs (15-30 million) were used for RNA extraction. Total RNA was isolated using an RNeasy mini kit (Qiagen, Germantown, MD). Genomic DNA was eliminated by RNase-free DNase I digestion (Qiagen) during the RNA isolation procedure. Isolated total RNA was analyzed on an Agilent 2100 Bio-analyzer with the use of the RNA 6000 Nano labchip kit (Agilent Technologies, Palo Alto, CA). First strand cDNA was synthesized using SuperScript RNase H$^-$ Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random primers (Invitrogen) according to the manufacturer's instructions.

Real-time PCR for CD63 mRNA quantification. Quantification of the *CD63* gene expression was conducted using TaqMan fluorescence-based, real-time PCR. We had previously identified ß-actin *(ACTB)* as a suitable reference gene for PMN expression studies (25). *CD63* and *ACTB* expression

Figure 1. Schematic overview of the 11 investigated polymorphisms in the *CD63* gene (NT_029419). The positions of these SNPs in the *CD63* gene sequence (+1 is defined as the start of transcription) are as following: -1523 (rs772254, C/T), -106 (rs12818008, C/T), 1361 (rs3138132, C/G), 2396 (rs2231467, A/G), 2820 (rs11574657, T/C), 3196 (rs1804040, G/A), 3410 (rs1804041, T/C), 3443 (rs1050043, G/A), 3845 (rs3138133 T/G), 3977 (rs3138134, T/C), and 8041 (rs3138144 C/G). A-adenine, C-cytosine, G-guanine, T-thymine, and rs-database SNP accession number.

kits were purchased from Applied Biosystems (Foster City, CA). The standard curves for *CD63* and *ACTB* were constructed from cloned PCR products using a TA cloning kit (Invitrogen). The 10X diluted cDNA samples from the study subjects were run in triplicate wells using 384-well plates on an ABI PRISM 7900HT Sequence detection system (Applied Biosystems). The PCR program was initiated at 95˚C for 15 min to activate the Taq DNA polymerase, followed by 40 thermal cycles of 15 sec at 94˚C and 1 min at 60˚C. The expression level of each subject was expressed as a ratio of *CD63* level over *ACTB* level.

Flow cytometry analysis of CD63 protein expression. In order to measure the level of CD63 expression, intact and permeabilized PMNs were subject to immunostaining and flow cytometry analysis. PMNs were permeabilized by electroporation immediately before use. As in previous studies (26-28) the electro-permeabilization procedure was carried out at room temperature. Cells (3-5x10^6) were resuspended in 0.6 ml electroporation buffer (120 mmol/l KCl, 10 mmol/l NaCl, 1 mmol/l KH_2PO_4, 10 mmol/l glucose, 20 mmol/l Hepes, pH 7.0), and transferred to a Gene Pulser cuvette (Bio-Rad) and subjected to 2 discharges of 5 kV/cm, 25 μF, and 50 Ω using Gene Pulser II (Bio-Rad). The cells were pipetted gently between pulses. Permeabilized cells were immediately transferred to a plastic tube and fixed with 5% formalin.

Aliquots of intact and permeabilized PMNs were incubated with 100 μl of 2.5 μg/ml mouse anti-human CD63 monoclonal antibody (Becton Dickinson, San Jose, CA) for 40 min at room temperature. Additional aliquots of intact and permeabilized PMNs were prepared and incubated with 2.5 μg/ml mouse anti-human IgG1 for 40 min to act as negative controls. After washing, PMNs were incubated with phycoerythrin-conjugated rabbit anti-mouse IgG (Santa Cruz Biotechnology Inc., Santa-Cruz, CA) for another 40 min. Finally, cells were analyzed by a FACScan flow cytometer (Becton Dickinson).

SNP selection and genotyping. The *CD63* gene is located on chromosome 12q12-q13 and has a length of 3.5 kb. A total of 11 putative polymorphisms in the *CD63* gene were investigated, including 2 promoter polymorphisms, 4 non-synonymous polymorphisms, 3 polymorphisms located in the 3'UTR, and 2 intronic SNPs located in the middle of the gene (Fig. 1). The average marker density was ~300 bp, sufficient to determine the linkage disequilibrium pattern of the gene. The genotypes were determined by PCR followed by restriction endonuclease digestion, allelic-specific PCR, or TaqMan genotyping assays. All the investigated polymorphisms were further analyzed by automatic sequencing to validate the genotyping results.

Statistical analysis. The correlations between each individual *CD63* polymorphism and gene expression and released MPO were evaluated with the use of the non-parametric Wilcoxon test. Bonferroni correction was made to correct for the multiple comparisons (corrected P-value = P-value x number of comparisons). All the statistical analysis was performed in JMP (SAS Institute Inc., Cary, NC). The level of significance was set at $P<0.05$.

Results

CD63 mRNA and protein were successfully measured. The threshold cycle values for *CD63* and *ACTB* were in the range of 20-22 cycles. There was *CD63* protein expression on the cell surface. However, a dramatic increase in CD63 staining was observed in permeabilized cells, which suggested that majority of the CD63 protein was located in the cytoplasm and/or on intracellular granules. The COPD patients had significantly lower *CD63* mRNA (0.37±0.01 vs. 0.51±0.04, P=0.008) and lower total CD63 protein (175.44±12.77 vs. 214.25±13.35, P=0.007) when compared with the healthy individuals (Fig. 2). In contrast, the patients had a higher level of surface *CD63* protein. The COPD patients also released less MPO than the healthy subjects (5.25±0.31 vs. 7.40±0.61, P=0.03) (Fig. 3).

Among the 11 investigated putative polymorphisms, 3 were polymorphic in our study cohort. They were -1523C/T (rs772254) in the promoter region, 1361C/G (rs3138132) in intron 1, and 8041C/G (rs3138144) in the 3' genomic region. The frequencies of the minor alleles for -1523C/T and 1361C/G polymorphisms were ~6%. The 8041C/G polymorphism was more prevalent with a minor allele frequency of ~48%. The observed genotype frequencies of the three polymorphisms conformed to that expected from Hardy-Weinberg equilibrium. The -1523C/T and 1361C/G alleles

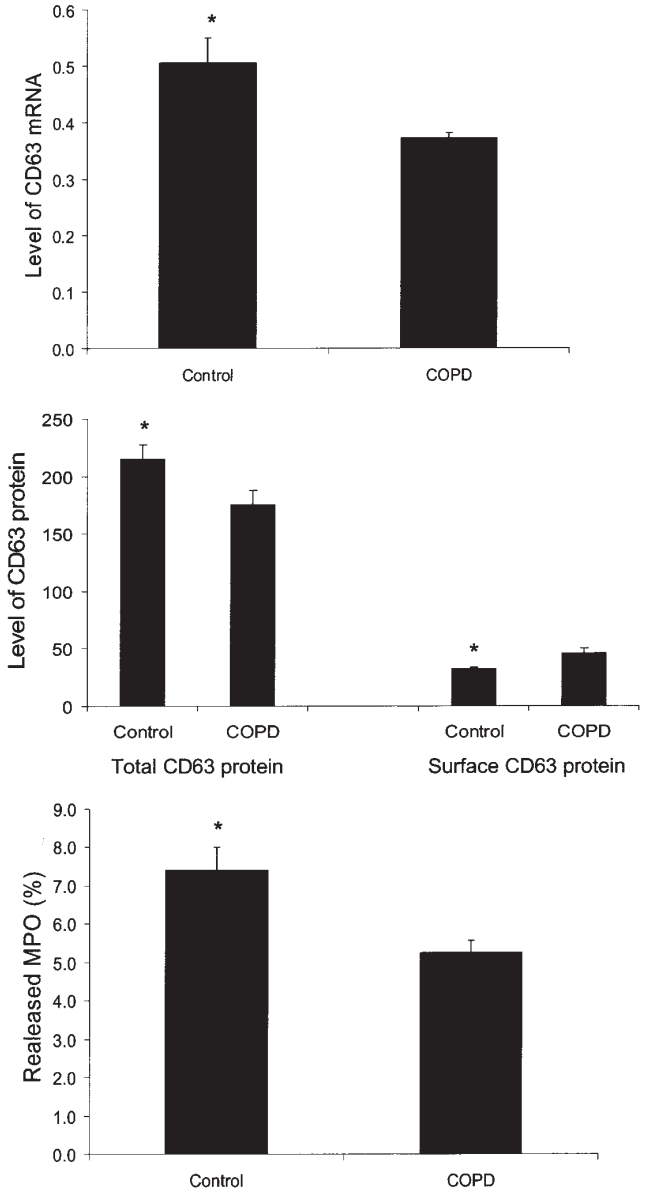

Figure 2. *CD63* expression and MPO release in the healthy subjects and COPD patients. The COPD patients had lower *CD63* mRNA (P=0.008) and lower total CD63 protein (P=0.007), and less released MPO (P=0.03) than the healthy individuals.

were significantly associated with each other (r^2=1, P<0.0001).

There was no association between the genetic polymorphisms and *CD63* expression at both the mRNA and protein levels in the healthy subjects (Table II). In the COPD patients 1361C/G was marginally associated with *CD63* mRNA level (P=0.04) when analyzed with the use of the non-parametric Wilcoxon test (Table III). However, after Bonferroni correction for multiple comparisons the P-value was no longer significant. The 8041C/G polymorphism was associated with MPO release in the healthy individuals, even after Bonferroni correction (Fig. 3). PMNs with the CC genotype released more MPO (9.05%±1.11%) compared with cells with the GG genotype (4.94%±0.68%) (P=0.007), which

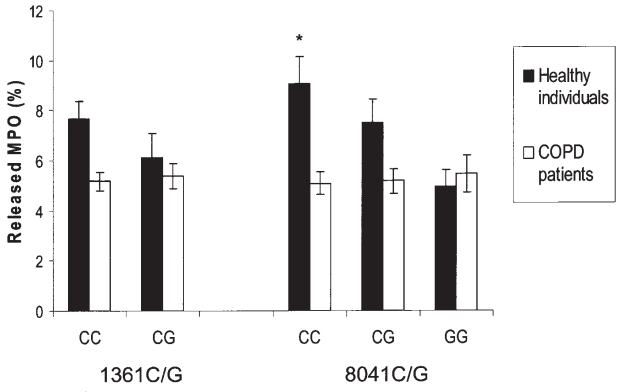

Figure 3. *CD63* genotypes and the amount of released MPO upon IL-8 stimulation in both healthy individuals and COPD patients. *8041C/G was associated with MPO release in the healthy individuals (P=0.007).

remained significant (P=0.021) after correction for the three outcome variables tested (mRNA level, protein level and MPO release). There was no association observed in the COPD patients with level of MPO release.

Discussion

PMNs play a crucial role in host defense. PMN hyperactivity and excessive mediator release have been implicated in the chronic inflammation associated with COPD. As a membrane marker of azurophilic granules, CD63 is involved in the process of azurophilic granule exocytosis in PMNs (16). However, its precise biological function in this process is unclear. Identifying genetic mutations which lead to loss-of-function or gain-of-function is one way to provide insight into the physiological function of the protein.

In this study, the gene expression experiments were designed to assess whether genetic polymorphisms of this critical molecule contribute to altered PMN function in all individuals or only in the specific context of COPD. We found the expression pattern of *CD63* in the COPD patients was dramatically distinct from that in the healthy individuals. The COPD patients had significantly lower *CD63* mRNA and lower *CD63* protein. PMNs from COPD patients also released less MPO compared with the cells from the healthy individuals. Although these two groups were not matched and therefore the observed difference is hard to interpret, the difference in expression implies that different factors regulated gene expression in the COPD patients than in the healthy subjects. Therefore, the effects of genetic polymorphisms on gene expression could be very different between these two groups, and this is the reason that we performed separate analysis of the groups.

The *CD63* gene spans 3.5 kb of the genomic region on chromosome 12q12-q13. There are >30 putative polymorphisms reported in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/SNP/). In general, polymorphisms located in the promoter region or introns of genes could affect the process of gene transcription or splicing, whereas polymorphisms in exons could result in amino acid changes and consequently affect the biological function of the protein. Both situations could lead

Table II. Genotypes and *CD63* expression in the healthy subjects.

		No.	*CD63* mRNA	Non-corrected P-value	CD63 protein	Non-corrected P-value
-1523C/T	CC	52	0.48±0.04	0.43	212±14	0.91
	CT	8	0.67±0.19		223±41	
1361C/G	CC	52	0.48±0.04	0.43	213±14	0.91
	CG	8	0.67±0.19		223±41	
8041C/G	CC	18	0.46±0.07	0.56	225±26	0.82
	CG	25	0.49±0.07		212±20	
	GG	17	0.58±0.09		207±25	

The mRNA level of *CD63* is expressed as the ratio of *CD63*/*ACTB*. The protein level of *CD63* is the mean fluorescence intensity (MFI) of CD63 staining. All the values listed are mean ± SE.

Table III. Genotypes and *CD63* expression level in the COPD patients.

		No.	*CD63* mRNA	Non-corrected P-value	CD63 protein	Non-corrected P-value
-1523C/T	CC	61	0.37±0.01	0.92	182±15	0.43
	CT	9	0.38±0.03		140±14	
1361C/G	CC	61	0.36±0.01	0.04	181±14	0.54
	CG	9	0.42±0.02		148±19	
8041C/G	CC	19	0.38±0.02	0.76	207±30	0.23
	CG	35	0.36±0.01		179±18	
	GG	16	0.38±0.02		137±15	

The mRNA level of *CD63* is expressed as the ratio of *CD63*/*ACTB*. The protein level of *CD63* is the mean fluorescence intensity (MFI) of CD63 staining. All the values listed are mean ± SE.

to changes in the total level of active protein in the cell. Herein, we investigated 11 putative genetic polymorphisms. However, most of the polymorphisms were monomorphic in our sample populations, which suggests that these polymorphisms are very rare in the Caucasian population or perhaps do not exist (i.e. represent sequencing errors). A study performed by another research group demonstrated similar results (29). This group sequenced all the exons of the *CD63* gene to look for mutations which could account for the pathogenesis of a rare autosomal recessive disorder Hermansky-Pudlak syndrome (HPS), and no mutations were detected. In addition, there are only three *CD63* polymorphisms reported in the latest release of the HapMap dataset (http://www.hapmap.org/) and none of them are polymorphic in the Caucasian study population. All these data indicate that there is a low level of genetic diversity in the *CD63* gene.

We did not observe any significant association of genetic polymorphisms with gene expression in either the healthy individuals or COPD patients. These results suggest that the influence of genetic variants on *CD63* expression is limited in PMNs. However, it is also possible that this study is not adequately powered to detect small-scale differences in gene expression. In addition, we acknowledge that gene expression is a net outcome of multiple, complicated regulatory processes, and other regulatory mechanisms must be present. Drucker *et al* reported that DNA methylation and chromatin remodelling significantly contributed to tetraspanin silencing in multiple myeloma (30). This study provided important clues that epigenetic modification might be critical in the regulation of *CD63* expression.

We also evaluated the correlation between genetic polymorphisms and azurophilic granule degranulation. The 8041C/G polymorphism, which is ~4 kb downstream of the *CD63* gene, was associated with MPO release in the healthy subjects even after Bonferroni correction. While this is a relatively small difference in terms of percentage of the total cellular MPO released it may have biological significance as it represents an ~2-fold difference in levels. Since 8041C/G was not associated with *CD63* expression, the association of 8041C/G with MPO release could be due to other reasons, such as linkage disequilibrium between 8041C/G and another functional polymorphism in a nearby gene, such as BLOCIS1,

which is involved in the biogenesis of PMN azurophilic granules (31).

In summary, we report that the CD63 gene has few polymorphisms and the influence of genetic polymorphism on gene expression is limited in the context of COPD, but its role in other diseases has yet to be determined. The numerous roles that CD63 plays in the cell make the functional 8041C/G polymorphism potentially of importance in many disease states.

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