No evidence of chromosome damage in chronic obstructive pulmonary disease.

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No evidence of chromosome damage in chronic obstructive pulmonary disease (COPD)

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Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality in industrialized countries. It is characterized by a progressive airflow limitation resulting from an abnormal inflammatory response of the lungs to inhaled gases and particles. Since oxidative stress is thought to play a role in COPD, and since increased oxidative stress is associated with chromosomal instability in several diseases, we investigated whether such relationship also exists in COPD. Whole blood lymphocytes from 49 COPD patients and 48 age- and sex-matched controls were cultivated *in vitro* and cytogenetic damage was evaluated by micronucleus (MN) and sister-chromatid-exchange (SCE) assays. In patients with COPD, MN frequency was not significantly different from that of controls. Similarly, SCE frequency did not differ in the two groups suggesting no disturbance in DNA replication. Unlike other diseases characterized by oxidative stress, COPD does not appear to be associated with DNA damage.

Introduction

Chronic obstructive pulmonary disease (COPD) is a major leading cause of chronic morbidity and mortality in industrialized countries, and is supposed to become the third leading cause of death and fifth cause of disability by the Year 2020 (1,2).

COPD is characterized by a progressive, not fully reversible, airflow limitation resulting from an abnormal inflammatory response of the lungs to particles and gases (2,3). The pathogenesis of COPD is not fully understood, but epidemiological studies have shown a strict correlation with environmental and host factors (3). The main environmental factor is exposure to cigarette smoke (CS). Yet, only a minority of smokers develop COPD suggesting that genetic factors may contribute to the development of the disease. As of now, alpha-1-antitrypsin (AAT) deficiency is the only recognized genetic predisposing factor, although it accounts for <5% of all the COPD cases (4,5).

Clinical studies clearly indicate a strong association between tobacco smoke-induced oxidative stress and inflammatory response in the lung (6–10), with a close relationship between severity of lung disease and cumulative smoking exposure (11). Inhaled oxidants and endogenous oxygen-derived free radicals are the main sources of the abnormal oxidative stress in COPD (12).

As an association between oxidative stress and chromosome instability has been reported in a variety of chronic diseases such as Parkinson's disease (13), coronary artery disease (14), Alzheimer disease (15), Raynaud's phenomenon (16), diabetes mellitus (17,18) and hypercholesterolemia (19), the present study was aimed at establishing whether genome DNA damage is also present in COPD. Cytogenetic endpoints are sensitive biomarkers that are widely accepted to evaluate chromosome damage (20–23). To this end, we measured the relative frequency of micronuclei (MN) and sister-chromatid-exchanges (SCE) in a group of patients with established COPD and in a matched group of non-diseased subjects.

Materials and methods

Subjects

The study sample consisted of 97 subjects who were evaluated at the outpatient clinic of our institution between November 27, 2002 and January 31, 2004. All subjects were examined uniformly according to a standardized protocol including clinical history, physical examination, spirometry and chest radiography. Criteria for patient recruitment were a firm diagnosis of COPD; airflow obstruction as manifested by a ratio of forced expiratory volume in one second (FEV_1) to forced vital capacity (FVC) $<70\%$ and $FEV_1 \leq 70\%$ of normal predicted values (6); no significant reversibility of airflow obstruction (change in $FEV_1 \leq 12\%$ after bronchodilator); smoking history ≥ 20 pack-years. Patients were excluded from the study if they had an established diagnosis of asthma, other obstructive syndromes, lung cancer, or if they had an acute exacerbation in the 4 weeks preceding recruitment.

The control group consisted of individuals who had no evidence of airflow obstruction ($FEV_1/FVC > 70\%$ and $FEV_1 \geq 80\%$ predicted). Controls were selected to match patients with COPD for age, gender and cumulative smoking history. None of them reported occupational exposure or history of chronic lung disease, and none had acute respiratory infection in the 4 weeks preceding the study entry.

On interviewing the subjects recruited to the study, care was taken to identify any comorbid condition which may *per se* induce genotoxic damage.

An informed written consent was obtained from all subjects, and the protocol was approved by the local ethics committee.

Cell culture

Peripheral blood was collected in heparinized tubes. Lymphocyte cultures were set up by mixing 0.5 ml of whole blood with 4.5 ml of RPMI-1640 medium containing 15% fetal calf serum, antibiotics (5 UI/ml penicillin and 5 μg/ml streptomycin) and phytohaemagglutinin (PHA, 1.5% of the final culture medium) (all reagents from Gibco, Life Technologies, Italy) according to standard techniques (24). Duplicate cultures were incubated at 37°C in a humidified atmosphere with 5% CO2 and maintained for 72 h.

MN test

Cytochalasin B (3 μg/ml, Sigma Chemical Co., St Louis, MO) was added after 44 h of culture to block cytokinesis, allowing identifying lymphocytes dividing in culture. Cells that have undergone the first mitosis are thus recognized as binucleated cells and are selectively screened for the presence of MN (25). Cell harvesting, hypotonic treatment, fixation and slide preparation were performed according to standard procedures (22,24). MN were scored blindly in 1000 binucleated cells, following standard criteria (26), and the frequency was expressed as the number of binucleated cells presenting one or more MN per 1000 cells. The nuclear division index (NDI) was calculated according to the formula NDI = [(Mn + 2Bn + 3Trn + 4Ten)/(Mn + Bn + Trn + Ten)],

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Table I. Baseline characteristics of the study sample

Characteristics	COPD (n = 49)	Controls (n = 48)
Sex		
Males, *n* (%)	44 (90)	42 (87)
Females, *n* (%)	5 (10)	6 (13)
Age (mean ± SD)	64.8 ± 8.1	65.7 ± 6.2
Smoking habit		
Pack-years (mean ± SD)	47.1 ± 16.1	42.3 ± 13.7
Current smokers, *n* (%)	27 (55)	25 (52)
Former smokers, *n* (%)	22 (45)	23 (48)
Lung function tests		
FEV_1/FVC % (mean ± SD)	52 ± 11	76 ± 4
FEV_1 % pred (mean ± SD)	53 ± 13	94 ± 12
Comorbidities		
None, *n* (%)	15 (31)	18 (37)
Hypercholesterolemia, *n* (%)	14 (29)	14 (29)
Ischemic heart disease, *n* (%)	6 (12)	17 (35)
Systemic arterial hypertension, *n* (%)	17 (35)	19 (40)
Other cardiovascular disorders, *n* (%)	9 (18)	4 (8)
Diabetes mellitus, *n* (%)	7 (14)	5 (10)
Medications		
None, *n* (%)	15 (31)	21 (44)
Bronchodilators, *n* (%)	19 (39)	0 (0)
Cardiovascular therapy, *n* (%)	24 (49)	26 (54)
Statines, *n* (%)	10 (29)	9 (19)

n represents the number of individuals presenting a certain factor. Values are expressed as number and percentage (in brackets), or as mean ± SD.

where Mn, Bn, Trn and Ten indicate the number of mono, bi, tri and tetranucleated cells, respectively.

SCE test

A total of 6 μg/ml of 5-bromodeoxy-uridine (BrdU; Sigma-Aldrich, Steinheim, Germany) were added after 24 h of culture and the FPG method was used to differentially stain sister chromatids (27). Colcemid (0.05 μg/ml; Sigma-Aldrich, Steinheim, Germany) was added during the last 2 h and a minimum of 30 well-stained second division metaphases was scored per individual.

The proliferation index (PI) was calculated from BrdU-labeled metaphases according to the formula $PI = [(M_1 + 2M_2 + 3M_3)/(M_1 + M_2 + M_3)]$, where M_1, M_2 and M_3 represent the number of first, second and third metaphases, respectively.

Statistical analysis

Differences between demographic and clinical characteristics were assessed between COPD patients and controls by contingency tables for categorical variables and χ^2-test. As the frequencies of MN and SCE were normally distributed both in the two diagnostic groups, parametric one-way analysis of variance and unpaired *t*-test were used to score the differences in the frequencies of MN and SCE among each category of the following variables: presence/absence of COPD, sex, smoking habits, medications and comorbidities. Pearson's coefficient was used in evaluating correlations between MN or SCE frequencies and continuous variables (age, FEV_1 and pack-years of smoking). Two-tailed *P*-values <0.05 were considered statistically significant throughout. Statistical analysis was performed with STATA 8 software (StataCorp, College Station, TX).

Results

The baseline characteristics of the study sample are reported in Table I.

There was no significant difference between patients with COPD and controls as regards age, gender and pack-years of smoking. Similarly, there was no difference between the two diagnostic groups as to the prevalence of comorbid conditions except for ischemic heart disease whose prevalence was significantly higher ($P < 0.05$) in controls than in COPD patients (Table I).

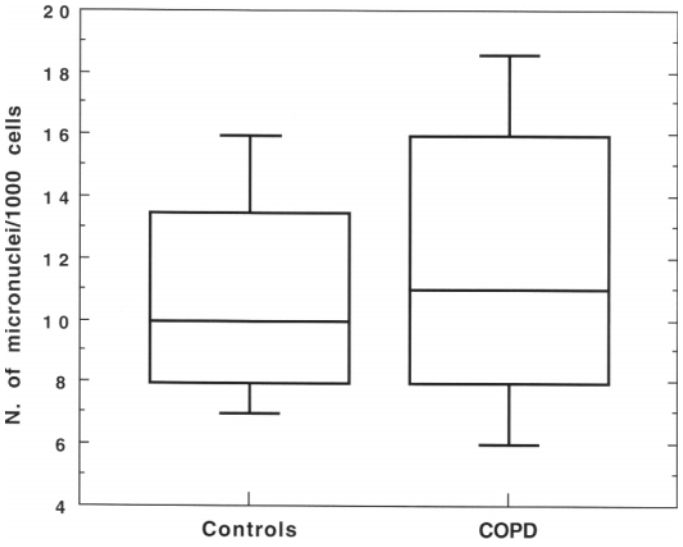

Fig. 1. Box-and-whiskers plot of the frequency distribution of MN in 49 patients with COPD and in 48 age- and sex-matched controls. Line in box is 50th percentile (median). Limits of box are 25th and 75th percentiles. Whiskers are 10th and 90th percentiles.

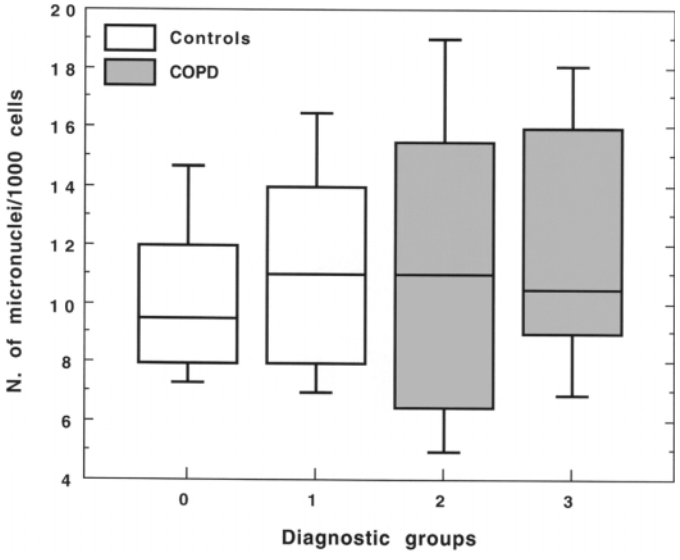

Fig. 2. Box-and-whiskers plot of the frequency distribution of MN in patients with COPD (stippled boxes) and in age- and sex-matched controls (open boxes). The two diagnostic groups are divided according to the presence or absence of comorbid conditions. Group 0: controls with no comorbidity; Group 1: controls with comorbidity; Group 2: COPD with no comorbidity; Group 3: COPD with comorbidity.

The frequency distribution of MN in the two diagnostic groups is displayed in Figure 1.

In patients with COPD, MN averaged 11.8 ± 4.6 and were not significantly different from those measured in controls (10.9 ± 3.6). To assess the effect of comorbidity on MN frequency, COPD patients and controls were further divided in two subgroups according to the presence or absence of comorbid conditions. As shown in Figure 2, there was no significant difference in MN frequency across the four subgroups. The number of MN averaged 11.7 ± 5.3 in patients with COPD and no comorbidity and 10.3 ± 3.1 in controls with no comorbidity ($P = 0.38$).

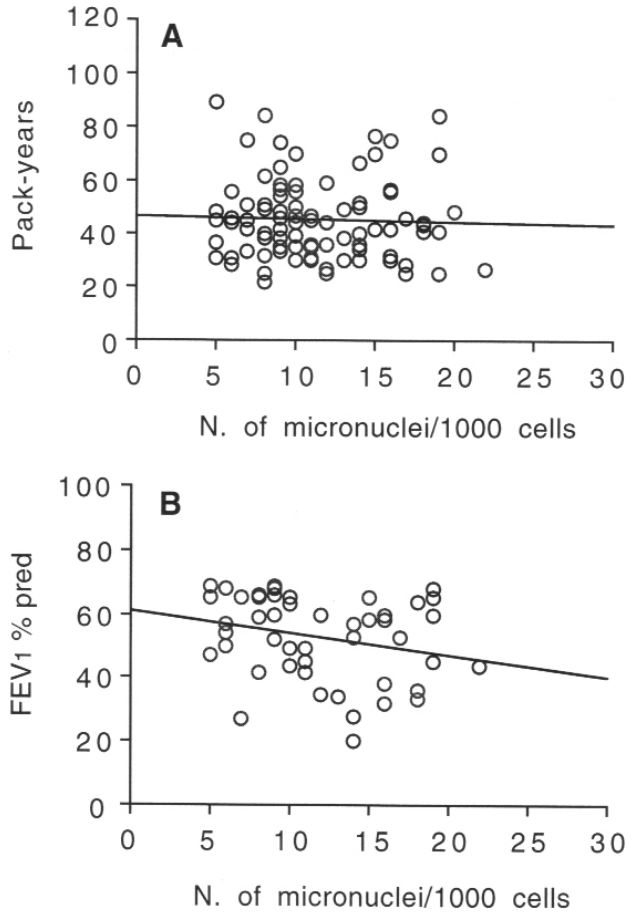

Fig. 3. (**A**) Relationship beween frequency of MN and pack-years of smoking in the whole study sample. (**B**) Relationship between frequency of MN and forced expiratory volume in one second (FEV_1) as percent of the predicted value in patients with COPD.

Considering the whole study sample, no significant relation was found between MN frequency and age ($r = 0.15$) or pack-years of smoking ($r = 0.07$; Figure 3A). In patients with COPD, there was no relationship between MN and degree of airflow obstruction as reflected by FEV_1 % predicted ($r = 0.25$; Figure 3B).

SCE analysis was performed and the PI calculated in a random subset of 48 individuals (25 COPD and 23 controls). SCE frequency did not differ in the two groups (4.24 ± 0.7 in COPD versus 4.89 ± 1.5 in controls), suggesting no disturbance in DNA replication. PI, calculated from SCE test, and NDI, calculated from MN test, had similar values in COPD and controls, suggesting that the status of the cell cultures and the capacity of cells to divide were the same in the two groups (data not shown).

Discussion

Monitoring DNA damage in pathologies is increasingly performed, as it may add another dimension to their clinical manifestation and could represent a potential target of therapeutical intervention.

Different kinds of DNA damage, ranging from oxidized bases to gross DNA changes, such as chromosome breaks which may drive the formation of MN, have been related to abnormal oxidative stress, which also underlie several pathologies (13–19). COPD is characterized by chronic oxidative stress as well, and very recently the presence of DNA damage, measured by the comet test, has been reported (28).

In our study, instead, using MN and SCE frequencies as biomarkers, we did not find any evidence of genomic alterations.

It has been reported that when oxidative stress is prolonged, cells may undergo apoptosis or direct necrosis (29,30) and recently, increased apoptosis have been demonstrated in lymphocytes obtained from the airways of COPD (31–33). Cells characterized by oxidative stress could give rise to MN, as a result of DNA damage, but would be also prone to be eliminated by an efficient apoptosis pathway, thus explaining our failure to find genomic damage in COPD patients.

We also failed to find any modulation of MN and SCE frequency by smoking habits: in fact there was no statistically significant difference in MN frequency between current and former smokers ($P = 0.29$). As the association between smoking and lung cancer has been firmly established (34), the genotoxic effects of CS in *in vitro*/*ex vivo* experiments are not so clear, despite the well known mutagenic and carcinogenic potential of many components of the CS.

In fact, many studies report unexpected negative results (35) and sometime smokers present lower MN frequency than non-smokers (36). Possible explanations for this lack of effects has been proposed, such as low sensitivity of MN test, lower effective exposure of lymphocytes to CS than other organs as lung, low survival of damaged cells (apoptosis or necrosis), delay in the cell cycle giving rise to false negative results (37). Our present data of PI and NDI seem to exclude this latter explanation.

A huge literature is also present on non-human systems. As recently reviewed by DeMarini (38), CS or its condensate is able to induce SCE and MN in rodent V79 and CHO cells *in vitro* and in the bone marrow, lung and erythrocytes of mice/rats after *in vivo* exposure to CS. Transplacental effects have also been reported: mice born of exposed mothers exhibit a high frequency of MN in the liver and blood cells (38,39).

Interactions may also occur among the >4000 CS components and protective substances may also mask the genotoxicity of this complex mixture: CS has been shown to inhibit the required metabolic activation of several mutagens (40), and the mutagenicity of three major nitrosamines in *Salmonell*a and rodent cells (41). Individual CS susceptibility may also be strongly modulated by genetic factors. It is well known the involvement of cytochrome P450 polymorphism in lung carcinogenesis: *CYP2D6* gene is suspected because of its activation of tobacco genotoxins (42), while genetic polymorphism of *CYP2A6* was associated with cancer susceptibility caused by smoking (43,44). A genetic characterization of COPD patients could help identifying the so called 'susceptible' smokers, who will develop irreversible COPD (45).

The multifactorial nature of COPD itself (where several genes, genetic polymorphisms, required therapies, multiple exposures to CS) may alter and modulate the phenotype of this pathology and can account for the absence of detectable genetic damage.

Also the elder age of individuals from our populations can be considered a further contributing factor: aging has been associated with a reduction of regenerative capacity of cells, and an increased rate of apoptosis has been observed in senescent endothelial cells (46).

Furthermore, COPD is a highly regional disease in its clinical manifestations (47) whereas MN and SCE assays are

performed in circulating lymphocytes, surrogate marker cells of systemic genotoxic stress, collected by non-invasive techniques. This approach is based on the hypothesis that the genetic damage in peripheral blood lymphocytes may reflect critical events/damages occurring in loco, possibly by stress-mediated processes like disruption of genomic integrity, inflammation and apoptosis. It can be hypothesised that COPD is not characterized by a systemic genotoxic stress and/or lymphocytes do not represent the proper biological system to evaluate DNA damage in this pathology.

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