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Original article

Bronchoalveolar lavage fluid alteration in antioxidant and inflammatory status in lung cancer patients

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ABSTRACT

Background: Increased oxidative and inflammatory markers have been reported in lung cancer patients, but relatively few studies have investigated the presence of antioxidants both in the local lung environment and in the systemic circulation. Furthermore, it is hypothesized that the immune system activation in vivo is regulated by the redox environment.
Objectives: To investigate local and systemically circulating antioxidant and inflammatory mediators in lung cancer patients and potential correlations between them.
Methods: Forty two male patients (mean age 65 ± 8 years) with primary lung cancer were studied. Sixteen age and smoking history matched male subjects without any evidence of malignancy served as controls. Total antioxidant status (TAS) and glutathione (GSH), as well as interleukin-1a (IL-1a), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) were measured in bronchoalveolar lavage fluid (BALF) and serum samples.
Results: A statistically significant increase of TAS and GSH in BALF was observed in lung cancer patients compared to healthy subjects (0.27 ± 0.24 vs. 0.12 ± 0.02 mmol/L, $p = 0.02$ and 7.56 ± 4.29 vs. 4.62 ± 2.23 μmol/L, $p = 0.01$ respectively). Statistically significant correlations in cancer patients were observed in BALF between TAS and a. IL-1α ($r = 0.87$, $p < 0.001$), b. IL-6 ($r = 0.52$, $p = 0.001$) and c. TNF-α ($r = 0.67$, $p < 0.001$).
Conclusions: Alteration in antioxidant and inflammatory mediator status was found in lung cancer patients both in serum and in BALF compared to healthy subjects matched for smoking history. Moreover, a positive correlation was observed between antioxidants and pro-inflammatory cytokines, but only locally and not systematically.

1. Introduction

An inflammatory response has been observed in lung cancer both locally and systematically. Several studies report alterations in serum concentrations of different cytokines in lung cancer patients with increased values for most of them [1,2]. Conflicting findings have been reported for the alveolar compartment by using measurements in bronchoalveolar lavage fluid (BALF) [3,4].

The contribution of reactive oxygen species (ROS) in cancer is well established. Several investigators have reported that oxidants may be involved in the early events of carcinogenesis, including transformation and promotion [5–7] and increased oxidative stress has been reported in lung cancer patients [8,9] through several mechanisms. One of these is a non-specific chronic activation of the immune system with an excessive production of pro-inflammatory cytokines, which in turn may increase the ROS production [10]. It is also speculated that the redox environment in lung lining fluids is a critical factor in triggering responses in the innate and adaptive immune systems of the lung [11].

Despite our increasing understanding of the possible mechanisms through which oxidative stress exerts a regulatory role in tumor growth and progression, it is unclear whether it results from an increased oxidant production or from a failure of antioxidant systems [12]. Changes in antioxidant activities have been found in lung carcinoma cell lines exposed to cytokines suggesting that inflammation in the lung may contribute to alterations in the antioxidant mechanisms and create an intracellular environment promoting DNA damage and leading to cancer [13]. Several alterations in antioxidant enzymes activity in lung cancer tissue have been observed [13,14] but relatively few studies have been conducted on locally expressed and systematically circulating antioxidants [8,15].

Hypothesizing that local and systemic inflammation observed in lung cancer patients might be attributed to alteration in antioxidant status due to increased oxidant burden, we investigated the local and systemic antioxidant status as assessed by measuring total antioxidant status (TAS) and reduced glutathione (GSH) in BALF and serum samples, as well as the status of local and systemic pro-inflammatory cytokines by measuring interleukin-1a (IL-1a), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) in the same samples. Correlations

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between levels of antioxidants and pro-inflammatory cytokines were also statistically explored.

2. Material and methods

2.1. Subjects

Subjects' characteristics are summarized in Table 1.

The study population consisted of 42 consecutive patients (mean age 65 ± 8 years), all males with previously undiagnosed and untreated disease, that was histologically proven to be primary lung cancer (squamous cell carcinoma: 22, adenocarcinoma: 7, small cell carcinoma: 10 and mixed type: 3). Sixteen age matched subjects (mean age, 62 ± 5 years), all males as well, who underwent bronchoscopy for haemoptysis without any final evidence of malignancy (chest X-Ray, CT scan and bronchoscopy) served as controls. The etiology of haemoptysis was upper or lower respiratory tract infection and bronchoscopy was performed more than 4 weeks later in order to exclude the effect of acute infection. All patients and control subjects were smokers with comparable cigarette consumption and comparable body mass index (Table 1).

Comprehensive medical history and examination were undertaken, as well as spirometry for measuring FEV_1, FVC, FEV_1/FVC before and after bronchodilation. Blood samples were collected for complete blood count and serum chemistries just before bronchoscopy. Exclusion criteria were: presence of coexisting medical illness and intake of medications, including those with potential antioxidative or anti-inflammatory properties, such as N-acetylcysteine, trimetazidine, cotrico-steroids or non-steroidal anti-inflammatory drugs, as well as antioxidant supplementation. Patients with a diagnosis of chronic obstructive pulmonary disease, using GOLD guidelines [16], were excluded from the study to avoid confusion in the interpretation of the results due to the increased oxidant burden that the above disease presents. In addition, to exclude any effect of acute smoke exposure, all patients were instructed to avoid smoking the day bronchoscopy was performed.

The study had the approval of the institutional ethics committee on human experimentation and all subjects gave written informed consent.

2.2. Bronchoalveolar lavage

Bronchoalveolar lavage was performed in all subjects, using fiberoptic bronchoscopy according to the ERS guidelines for measurement of acellular components and recommendations for standardization of bronchoalveolar lavage [17]. The bronchoscope was wedged in a segmental bronchus of the middle lobe or lingula of the lung with endobronchial or chest-roentgenographic abnormalities. Four 50 mL aliquots of warmed normal saline were instilled and returned by gentle hand aspiration. Between 60% and 80% of instilled solution was routinely recovered without significant differences in the percentage of recovery between the control subjects and patients with cancer. Supernatant and serum samples were stored at −80 °C. All measurements were made in unconcentrated BALF.

Table 1
Subjects' characteristics.

	Controls	Cancer patients
Age (years)	62 ± 5 (45–77)	65 ± 8 (43–80)
BMI (kg/m^2)	21.6 ± 1.84 (19–24)	21.2 ± 2.17 (17–26)
FEV_1 (% pred)	95.0 ± 3.85 (89–100)	93.8 ± 5.4 (83–106)
Smoking habit (pack–years)	53.1 ± 10.9 (40–65)	56.7 ± 9.5 (35–92)

Data are presented as mean (SD) with ranges in parenthesis.
BMI: body mass index, FEV1: forced expiratory flow in one second.

2.3. Antioxidant measurements

Serum TAS was measured using a kit from Randox Ltd., Crumlin, UK, based on a colorimetric method as previously described [18]. The same method was used for measurements made in BALF [19]. By this method, incubation of 2,2′-azinobis-3-ethylbenothiazoline 6-sulphonate (ABTS®) with a peroxide (metmyoglobin) results in production of the radical cation $ABTS^+$. This species is blue-green in color, and can be detected at 600 nm. Antioxidants in the added sample cause inhibition of this color production to a degree that is proportional to their concentration. A Daytona RX, Randox, analyzer was used for the measurement and the reagent was calibrated with the standards contained in the kit.

The determination of GSH was done with a colorimetric assay (GSH-400, Oxis, USA) based on a nonenzymatic chemical reaction that proceeds in two steps as previously described [15]. Briefly, the first step induces the formation of thioether molecules between a patent reagent and all mercaptans present in the sample. In the second step, a β-elimination reaction under alkaline conditions induces the transformation of the GSH-thioether product into a chromophoric thione. The GSH concentrations were measured spectrophotometrically at a wavelength of 400 nm and compared with a standard curve of five distinct concentrations of standard GSH.

2.4. Cytokine measurements

The various human cytokines were measured with commercial kits (R&D systems Europe, Abingdon, OX 143 NB, UK) as previously described [1,2]. Briefly, a monoclonal antibody specific for each of the aforementioned cytokines has been precoated onto microplates. Samples were pipetted into the wells and any cytokine present was bound by the immobilized antibodies. Following a wash, an enzyme (HRP)-linked specific polyclonal antibody was added to the wells. Following a second wash, a substrate solution (H_2O_2/TNB) was added to the wells and color developed in proportion to the amount of cytokine bound in the initial step. The color development was stopped with sulfuric acid and the intensity of the color was measured in 450 nm.

3. Statistical analysis

The Statistical Program for Social Science (SPSS version 13) was used. Results are expressed as mean ± SD. Unpaired t-test was used for the comparison in measurements of antioxidants and cytokines between control subjects and cancer patients. Correlations between antioxidants and cytokines both in healthy controls and in cancer patients were estimated by calculating the Pearson's linear correlation coefficient (for each test the conventional p value <0.05 was lowered to $p<0.0033$ divided by 15, which is the number of comparisons, following the Bonferonni adjustment).

4. Results

Measured titers of antioxidants and cytokines in serum and BALF, both in healthy controls and cancer patients are shown in Table 2. Data are presented as mean (SD) with ranges in parenthesis.

4.1. Antioxidants

Significant increases of TAS and GSH in BALF were observed in lung cancer patients compared to healthy controls (0.12 ± 0.02 vs. 0.27 ± 0.24 mmol/L, $p=0.02$ and 4.62 ± 2.23 vs. 7.56 ± 4.29 mmol/L, $p=0.01$, respectively). On the other hand we were unable to find any significant differences among serum antioxidants,namely TAS and GSH.