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Glutamate-cysteine ligase modulatory subunit in BAL alveolar macrophages of healthy smokers

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Glutamate-cysteine ligase modulatory subunit in BAL alveolar macrophages of healthy smokers. C. Neurohr, A-G. Lenz, I. Ding, H. Leuchte, T. Kolbe, J. Behr. ©ERS Journals Ltd 2003.

ABSTRACT: The antioxidant glutathione (GSH) is increased in the epithelial lining fluid (ELF) of chronic smokers. The rate-limiting enzyme in GSH synthesis is glutamate-cysteine ligase (GCL), also known as γ-glutamylcysteine synthetase, consisting of a heavy, catalytic (GCLC) and a light, modulatory (GCLM) subunit.

To determine the contribution of bronchoalveolar lavage (BAL) cells to GSH levels in ELF, BAL was performed in eight smokers and eight never-smokers. Intra- and extracellular total glutathione (GSH_t) levels and GCL subunit expression were assessed.

GSH_t was increased in ELF from smokers (1,090.1±163.0 μM *versus* 559.2± 48.2 μM). GSH_t content of BAL cells (nmol·mg protein^{-1}) was decreased in smokers without differences reaching statistical significance (8.0±1.4 *versus* 12.4±2.6). GCLM expression was also reduced in smokers (0.6±0.1 versus 2.8±0.4) and correlated with intracellular GSH_t content. There was no significant difference in GCLC expression and in differential cell counts in BAL fluid.

The authors conclude that smoking does increase glutathione levels in the epithelial lining fluid but not intracellular levels in bronchoalveolar lavage cells. The data suggest that the intracellular glutathione concentration of bronchoalveolar lavage cells (predominately alveolar macrophages) is regulated by the modulatory glutamate-cysteine ligase subunit rather than the catalytic subunit.

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Cigarette smoking is the most clearly recognised factor leading to the development of chronic bronchitis and chronic obstructive pulmonary disease (COPD), which ranks as the fourth-leading cause of death in industrialised countries [1, 2]. COPD is an inflammatory disease, characterised by an oxidant/antioxidant imbalance as a major cause of respiratory cell damage [3, 4]. Cigarette smoke contains 10^{14}–10^{16} free radicals per puff and exposes the lung to an excessive oxidative burden [5].

The ubiquitous essential tripeptide glutathione (GSH; L-γ-glutamyl-L-cysteinyl-glycine) is a key intra- and extracellular-reducing agent protecting against oxidative stresses. Hence, GSH serves as one of the fundamental antioxidative defence mechanisms in the control of inflammatory processes in lung injury [6]. The rate-limiting enzyme in *de novo* GSH synthesis is glutamate-cysteine ligase (GCL), also known as γ-glutamylcysteine synthetase. GCL consists of a catalytic heavy subunit (GCLC) and a modulatory light subunit (GCLM) [7]. Both the GCLC and the GCLM genes contain antioxidant response elements, also referred to as electrophile response elements, for transcription factors (*i.e.* nuclear factor-κB, activator protein-1) necessary for GCL expression in response to diverse stimuli [8, 9]. It has been suggested that oxidants, antioxidants, and inflammatory and anti-inflammatory agents modulate the inducible and constitutive expression of GCL by the activation of these redox-sensitive transcription factors [10].

The normal lung epithelial lining fluid (ELF) contains high levels of GSH, which may be critical in protecting epithelial cells from smoke-induced oxidant injury [11]. Alterations in ELF GSH levels have been shown in various inflammatory conditions. For example, GSH is decreased in the ELF of idiopathic pulmonary fibrosis [12], acute respiratory distress syndrome [13] and human immunodeficiency virus-positive patients [14]. However, in the ELF of chronic smokers and COPD patients, GSH is present in increased concentrations [11, 15, 16]. Among the cell types known to export GSH are mononuclear phagocytes, lymphocytes, type-I and -II pneumocytes and fibroblasts, cells present in the lower respiratory tract and, in part, in bronchoalveolar lavage fluid (BALF) [17–19]. How these high extracellular GSH levels are established and how the mechanisms for increases or decreases are modulated is still not fully understood.

To evaluate the contribution of BALF cells to the elevated levels of ELF GSH in chronic smokers, intra- and extracellular GSH levels were investigated in the BALF of healthy chronic smokers and never-smokers. In addition, bronchoalveolar lavage (BAL) cells were analysed for messenger (m) ribonucleic acid (RNA) expression of GCLC and GCLM by using semiquantitative reverse transcriptase (RT)-polymerase chain reaction (PCR).

Methods

Subjects

Sixteen adult volunteers (eight active smokers and eight never-smokers with normal pulmonary function and chest

radiograph) were recruited to the study, which was approved by the local Ethics Committee. Informed written consent was obtained from each subject. The physical characteristics of the two groups are shown in table 1. No individual had taken oral or inhaled corticosteroids or bronchodilators for $\geqslant 6$ months before the study or did smoke during 12 h prior to the BAL.

Pulmonary function tests

For lung function tests, standard equipment (Jaeger & Thoennies, Würzburg, Germany) was used. Lung volumes were referenced to standard values as published by the European Community for Steel and Coal [20]. Blood-gas analysis was performed with arterialised capillary blood from the ear lobe (double values).

Biological samples

Serum and BALF were obtained by standard techniques, as described previously [21]. A fibreoptic bronchoscope was wedged in a subsegmental bronchus, and five serial infusions and aspirations with a 20-s suction period were performed, each of 20 mL sterile saline (0.9% sodium chloride). The recovered fluid was pooled and filtered through sterile gauze. Aliquots of the BALF were taken for GSH assays (see below), for total cell counts (Coulter counter; Coulter Electronics GmbH, Krefeld, Germany) and for cytocentrifuge preparations for differential counts. Viability was measured using the trypan blue exclusion method. The cells were pelleted and used for GSH assays or stored at -80°C until RNA extraction was performed, as described below. The supernatant was used to assess the amount of ELF.

Estimation of respiratory epithelial lining fluid

The volume of ELF was estimated by the urea dilution method [22]. Concentrations of urea in BALF and serum were measured with the urea nitrogen 65-UV kit (Sigma, St Louis, MO, USA or Serva, Heidelberg, Germany), and the dilution factor obtained was used to calculate GSH concentrations in ELF.

Glutathione concentration and form

GSH concentrations in BALF and bronchoalveolar cells were measured using the standard techniques as reported previously, with only minor modifications [23]. All determinations were made in triplicate and the average value was calculated.

Total GSH (GSHt) was calculated by GSH+(2×oxidised glutathione; GSSG) in BALF immediately after the BAL; 100 μL of the BALF supernatant (3,000×*g* for 10 min) was mixed with 1.1 mL of 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM ethylenediamine-tetraacetic acid (EDTA), 0.2 mM nicotinamide adenine dinucleotide phosphate (NADPH), 63.5 μM 5.5′-dithiobis(2-nitrobenzoic acid) (DNTB) and 4 U·mL^{-1} GSH reductase (all Sigma Chemicals Co.). The rate of reduction of DNTB was recorded spectrophotometrically at a wavelength of 412 nm.

For the measurement of intracellular GSHt in bronchoalveolar cells, the supernatant was removed after centrifugation (see above) and the cells were resuspended in phosphate-buffered saline. An aliquot of 1×10^6 bronchoalveolar cells was lysed by incubation in 1 mL of distilled water for 5 min. After centrifugation (3,000×*g* for 10 min) 100 μL of the lysate was assayed and the rate of reduction of DNTB was determined, as described above.

The GSHt concentration of the BALF and the bronchoalveolar cell lysate sample was calculated using an internal standard of 0.84 μM GSH [24]. The GSHt content of BAL cells is expressed as nmol·mg protein^{-1}. The protein levels of the BAL cells were assessed using the method of LOWRY *et al.* [25].

GSSG (*i.e.* glutathione disulphide) was determined using the method described by ADAMS *et al.* [26]. After centrifugation (3,000×*g* for 5 min), BALF supernatant was mixed with an equal volume of 10 mM *N*-ethylmaleimide in 0.1 M potassium phosphate buffer, pH 6.5, containing 17.5 mM EDTA. A total of 250 μL of the mixture was passed through a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA, USA) that had been prewashed with 3 mL methanol followed by 3 mL of aqua bidest. GSSG was eluted from the column with 1 mL of 0.1 M potassium phosphate buffer, pH 7.5, 5 mM EDTA. A total of 750 μL of the eluate was added to 250 μL of 0.1 M potassium phosphate buffer, pH 7.5, with 5 mM EDTA, 800 mM DTNB, 2 U·mL^{-1} GSH reductase and 1 mM NADPH. The rate of reduction of DTNB was recorded spectrophotometrically at 412 nm. Standards of GSSG (Boehringer, Mannheim, Germany) of known concentrations (0.25–4 μM) were processed exactly as the BALF samples and were used to generate standard curves. The concentration of reduced GSH was calculated by the following:

$$GSH = GSH_t - (2 \times GSSG) \quad (1)$$

Glutamate-cysteine ligase heavy, catalytic and light, modulatory subunit messenger ribonucleic acid expression

Ribonucleic acid extraction. Frozen BAL cells were lysed in ice cold TRIZOL reagent (GIBCO, Eggenstein, Germany). Total RNA was extracted according to the methods recommended by the manufacturer and redissolved in water. Total RNA yield was calculated by measuring the absorbance at 260 and 280 nm (assuming that A_{260} of 1=40 μg RNA). RNA integrity was judged by determining the ratio of A_{260}/A_{280}. Only probes with an A_{260}/A_{280} ratio from 1.6–2.0 were used for the following experiments.

First-strand complementary deoxyribonucleic acid synthesis by reverse transcription. A sample of 1.5 μg RNA in 12 μL ribonuclease (RNAse)-free water and 1 μL of oligo(dT)$_{12–18}$ (50 ng·μL^{-1}) was preheated to 70°C for 10 min and chilled on ice for 1 min. Of the reaction mixture, 7 μL, consisting of 2 μL

Table 1. – Physical characteristics of the two groups of volunteers

	Never-smokers	Smokers	p-value
F:M	6:2	2:6	
Age yrs	35.75±6.0	33.88±6.2	NS
VC % pred	111.5±5.3	108.8±5.3	NS
FEV1 % pred	113.1±5.6	107.3±4.5	NS
P_{a,O_2} kPa	11.3±0.4	11.1±0.4	NS
Pack-yrs		8.0±2.4	0.01
Cigarettes·day^{-1}		14.25±2.8	0.01

Data are presented as mean±SEM. F: female; M: male; VC: vital capacity; % pred: % predicted; FEV1: forced expiratory volume in one second; P_{a,O_2}: arterial oxygen tension. NS: nonsignificant.

RNAse-free PCR buffer, pH 8.4, containing 20 mM Tris-HCl and 50 mM KCl, 2 μL 25 mM $MgCl_2$, 1 μL 10 mM DNTB and 2 μL 0.1 M dithiothreitol, were added to the RNA/primer mixture. After 5 min of preincubation at 42°C, 1 μL (200 U) of Superscript II reverse transcriptase (GIBCO) was added, and RT for first complementary (c) deoxyribonucleic acid (DNA) was carried out over 50 min at 42°C. The reaction was terminated at 70°C for 10 min, followed by 1 min of chilling on ice. RNAse H (1 μL (2 U); GIBCO) was added and followed by incubation for 20 min at 37°C to digest the mRNA strand of the formed mRNA/DNA heteroduplex. The first-strand of cDNA was stored at -80°C.

Semiquantitative polymerase chain reaction. A sample of 1.5 μL cDNA was used for each PCR reaction. The primer set used for the amplification of GCLM, GCLC and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows:

GCLM: forward 5′-CAG CGA GGA GCT TCA TGA TTG-3′; reverse 5′-TGA TCA CAG AAT CCA GCT GTG C-3′ (size of PCR product: 241 base pairs (bp)).

GCLC: forward 5′-GTT CTT GAA ACT CTG CAA GAG AAG-3′; reverse 5′-ATG GAG ATG GTG TAT TCT TGT CC-3′ (size of PCR product: 383 bp).

GAPDH: forward 5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′; reverse 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′ (size of PCR product: 900 bp).

Each 50-μL reaction mixture consisted of 5 μL of 10×PCR buffer, 3 μL $MgCl_2$ (~1.5 mM), 1 μL of 10 mM DNTB mix, 1 μL of specific primer (~10 μM) for GAPDH, GCLM, GCLC (synthesised by MWG-Biotech, Ebersberg, Germany), 0.5 μL of Taq DNA polymerase (~2 U; GIBCO) and 37.5 μL of water. The cycles (RoboCycler Gradient 40 with hot top; Stratagene, Heidelberg, Germany) used were as follows: GAPDH 94°C for 3 min/94°C for 45 s/60°C for 45 s/72°C for 1 min for 24 cycles, followed by an extension step of 10 min at 72°C. The same cycle conditions were used for GCLM and GCLC but with an annealing temperature of 58°C for 29 PCR cycles.

Products were electrophoresed on a 2% agarose gel and viewed using a 300-nm ultraviolet transilluminator (Cybertech, Berlin, Germany) (fig. 1a). Samples from RT reactions that did not contain RT served as negative controls.

For quantification, PCR bands were stained with ethidium bromide (Sigma, Munich, Germany) and signal intensity was measured with an ultraviolet densitometer (Cybertech, Berlin, Germany). Densitometric values are expressed as the ratio of GCLM/GAPDH and GCLC/GAPDH (fig. 1b).

Statistics

Data are expressed as mean±SEM. For group comparisons, the nonparametric Mann-Whitney test was used. Correlations were calculated according to Pearson. Values of $p<0.05$ were considered significant.

Results

Bronchoalveolar lavage cell counts

Relative and absolute differential cell counts in BALF are shown in table 2. No significant differences between smokers and never-smokers could be found for these parameters. The absolute number of alveolar macrophages was elevated in the smokers′ group, although the differences did not reach statistical significance (p=0.25).

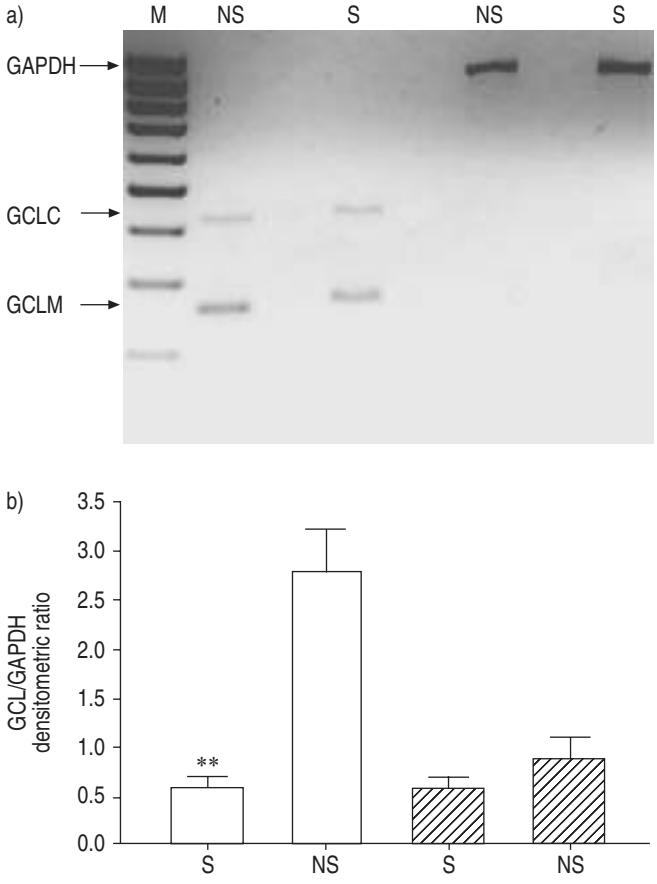

Fig. 1.–a) Reverse transcriptase-polymerase chain reaction (RT-PCR) of total ribonucleic acid (RNA) of bronchoalveolar lavage (BAL) cells. RT-PCR products for glutamate-cysteine ligase heavy, catalytic (GCLC) and light, modulatory (GCLM) subunits, and glyceraldehyde-3-phosphate dehydrogenase of smokers (S) and never-smokers (NS) are shown. Lane M contains deoxyribonucleic acid molecular size standard marker. b) Mean±SEM evaluation of GCLM (□) and GCLC (▨) messenger RNA expression in BAL cells of smokers (n=8) and never-smokers (n=8). **: $p<0.01$.

Glutathione concentrations

The GSHt concentration in native BALF was increased in smokers compared with never-smokers (10.8±1.4 *versus* 3.9±0.4 μM, respectively, $p<0.05$). This difference was even more pronounced when the respective GSHt concentrations in ELF were calculated (1,090.1±163 *versus* 559.2±48.2 μM,

Table 2.–Cell counts in smokers and never-smokers

	Never-smokers	Smokers	p-value
Recovery %	38.9±3.0	33.3±2.7	NS
Cell count $\times 10^6$	7.1±0.7	11.2±3.2	NS
Viability %	72.1±5.1	79.1±5.6	NS
AM %	93.3±2.7	94.9±2.3	NS
Neutrophils %	2.3±1.0	2.3±0.9	NS
Eosinophils %	0.9±0.7	1.3±0.6	NS
Lymphocytes %	3.6±1.7	1.5±1.1	NS
AM 10^4·mL $BALF^{-1}$	7.9±1.5	14.9±3.9	NS
Neutrophils 10^4·mL $BALF^{-1}$	0.2±0.07	0.3±0.1	NS
Eosinophils 10^4·mL $BALF^{-1}$	0.04±0.03	0.09±0.04	NS
Lymphocytes 10^4·mL $BALF^{-1}$	0.2±0.1	0.3±0.2	NS

Data are presented as mean±SEM. AM: alveolar macrophages; BALF: bronchoalveolar lavage fluid. NS: nonsignificant.

respectively, p<0.005; fig. 2). The concentration of the reduced form of GSH was also increased in smokers, both in native BALF (9.3±1.1 *versus* 3.4±0.3 μM, p<0.001) and in ELF (963.6±155.8 *versus* 508.7±46.3 μM, p<0.005; fig. 2). Accordingly, the concentration of the oxidised (GSSG) form of GSH was decreased in the ELF of never-smokers (63.3±15.6 *versus* 25±3.4 μM, p<0.005; fig. 2). However, the ratio of the GSSG to the GSHt concentration (expressed as a per cent of GSHt) did not differ significantly between the smoker and never-smoker groups (4.7±0.7 *versus* 5.9±1.4%, respectively, NS).

The intracellular GSHt content of BAL cells was decreased in smokers compared with never-smokers, although the differences did not reach statistical significance (8.0±1.4 *versus* 12.4±2.6 nmol·mg protein^{-1}, respectively, p=0.081; fig. 3). There was no significant correlation between intracellular GSHt content and extracellular GSHt concentration in ELF (r=0.031, p=0.91).

Glutamate-cysteine ligase heavy, catalytic and light, modulatory subunit expression

The GCLM mRNA expression in BAL cells was significantly decreased in smokers compared with never-smokers (0.6±0.1 *versus* 2.8±0.4, respectively, p<0.01; fig. 1) and correlated with intracellular GSHt content (r=0.88, p<0.01; fig. 4).

For GCLC, a trend toward lower mRNA expression in the smoker group was observed, without the differences reaching statistical significance (0.6±0.1 *versus* 0.9±0.2, NS; fig. 1b).

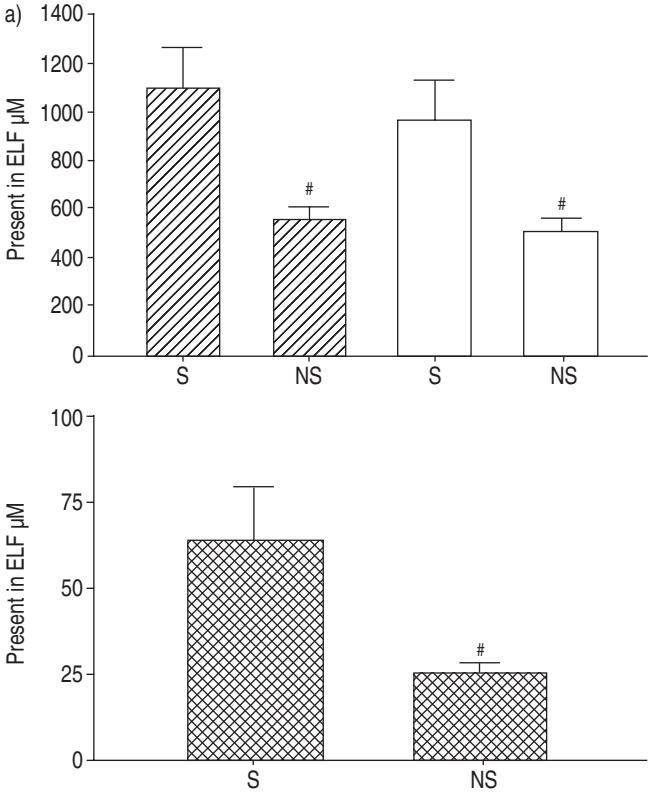

Fig. 2. – Comparison of the mean±SEM concentrations of a) total glutathione (▨), reduced glutathione (□), and b) oxidised glutathione (■) in epithelial lining fluid (ELF) of smokers (S; n=8) and never-smokers (NS; n=8). #: p<0.005.

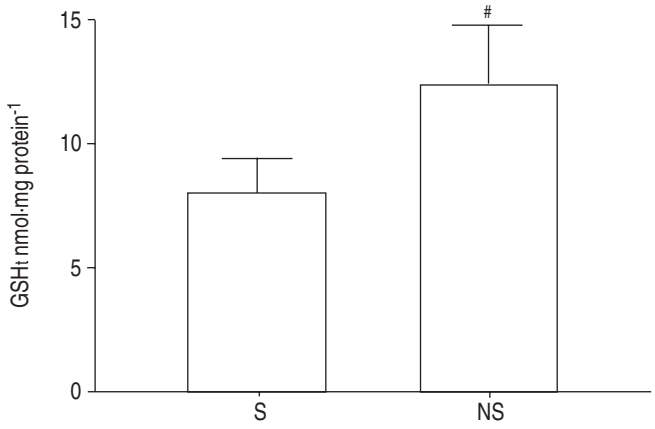

Fig. 3. – Comparison of mean±SEM intracellular total glutathione (GSHt) of smokers (S; n=8) and never-smokers (NS; n=8). #: p=0.081.

Discussion

GSH is a sulphydryl-containing tripeptide that plays a critical role in defending cells against oxidative stress in cigarette smoke-induced airways disease [6]. Several studies have shown significantly increased levels of GSH in ELF of chronic smokers compared with nonsmokers [11, 15, 27]. The present study is consistent with these findings and the evaluation of the ELF of healthy chronic smokers indicates significantly higher levels of total, reduced GSH and GSSG than in the never-smokers′ group. In both groups the GSH reductase reaction is driven strongly in favour of GSH, with a physiological GSH-to-GSSG ratio >90% [6]. The high levels of GSH observed in ELF of cigarette smokers have been interpreted as an adaptive defence mechanism of the lungs of chronic smokers. Recently, it has been shown that high GSH levels in cigarette smokers decrease epithelial permeability and protect alveolar epithelial cells from injury [28, 29].

The origin of lung ELF GSH is not yet known and it may come from a variety of sources. Simple diffusion from the plasma is unlikely because blood levels of GSH are 100-times lower than those in ELF, so that GSH has to be synthesised intracellularly and transported out of cells [30]. The alveolar macrophage is the predominating cell type in the BAL of smokers [31], and alveolar macrophages are supposed to be a

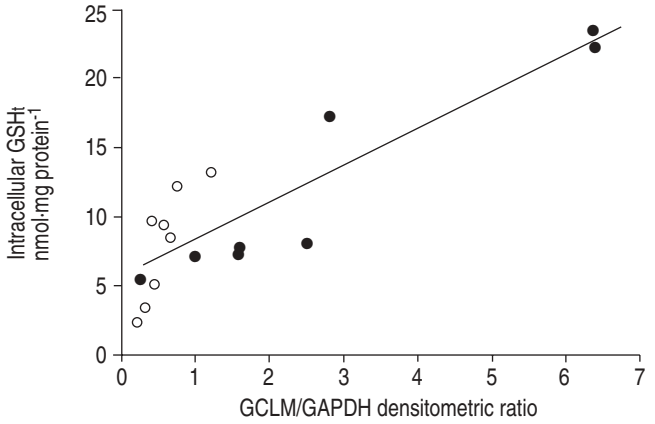

Fig. 4. – Correlation between light, modulatory glutamate-cysteine ligase subunit (GCLM) messenger ribonucleic acid expression and total intracellular glutathione (GSHt) of bronchoalveolar lavage cells (r=0.88, p<0.01). ○: smokers (n=8); ●: never-smokers (n=8). GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

major source of GSH [32]. In the current study, no significant differences in the composition of BAL cell populations and viability were detected between the two study groups. There was, however, an expected trend to elevated macrophage numbers in the smokers′ group [32]. More than 93% of the recovered cells from BAL were alveolar macrophages without statistically significant differences between both groups. With the use of flow cytometry-based separation techniques, only a minor increase in the purity of the alveolar macrophage population (up to 96%) could have been achieved [33]. Therefore, the present authors claim that the results obtained in this study primarily apply to the alveolar macrophage population. They found that the total intracellular GSH content of BAL cells, *i.e.* alveolar macrophages, was decreased in smokers without the differences reaching statistical significance, and they did not correlate with the extracellular GSH concentrations.

Rapid depletion of intracellular GSH has been shown to occur with exposure to cigarette smoke in epithelial cells *in vitro* and in rat lungs *in vivo* [28, 34]. This is followed by a sustained increase in GSH at 12–24 h in lungs *in vivo*, and in human alveolar and bronchial epithelial and endothelial cells *in vitro*, which is associated with an increased expression of mRNA for the GCL subunit genes for GSH synthesis [35–37]. However, in the present study, the authors found a significant decrease of GCLM mRNA expression in the BAL cells of smoking subjects compared with never-smokers. This reduction correlated with the diminished total intracellular GSH content in the smokers′ group. For GCLC there was a trend to reduced mRNA expression in smokers, without the differences reaching statistical significance. Since most studies of GCL gene regulation have focused on the catalytic subunit in *in-vitro* models of isolated cells [38, 39], oxidative stress seems to impose a condition under which transcription of GCLM also plays a critical role for the regulation of GCL activity [40, 41].

The GSH redox system is crucial in maintaining intracellular GSH homeostasis, using GSH as a substrate in the detoxification of peroxides involving glutathione peroxidase and leading to the generation of GSSG. Physiologically, glutathione reductase generation is driven strongly in favour of GSH, with the GSH-to-GSSG ratio normally >90%. If oxidant stress alters the GSH-to-GSSG ratio, this shift influences a variety of cellular processes, such as activation of transcription factors leading to upregulation of GCL. Due to the fact that the present authors did not differentiate between intracellular reduced GSH and GSSG, the data cannot exclude an intracellular shift of the dynamic GSH-to-GSSG ratio. Furthermore, an oxidative activation could eventually lead to the export of GSSG and a reduction of intracellular GSH stores. However, it is undisputed that GCL gene regulation plays a key role in maintenance of intracellular GSH in lung cells [6]. To the best of the authors′ knowledge, this is the first study reporting data on GCL gene expression in BAL cells of smokers and never-smokers.

The recent results of HARJU *et al.* [42] suggest that the expression of GCL is reduced in alveolar macrophages of healthy smokers compared with nonsmokers. Using immunohistochemistry, HARJU *et al.* [42] demonstrated a higher expression of both GCLM and GCLC subunits in alveolar macrophages in lung tissue samples of nonsmokers compared with smokers. These findings are in line with the current observations of a significantly decreased GCLM mRNA expression and a trend for lower GCLC mRNA expression in BAL cells in the smokers′ group. Reasons for the differences in GCLC expression not reaching statistical significance may be due to sample size and the relative short smoking history of the investigated volunteers compared with the study of HARJU *et al.* [42]. Furthermore, there is not necessarily a strong correlation between mRNA levels and the enzyme protein. Moreover, as observed by HARJU *et al.* [42], the GCL expression may also be different at various levels of the airways and in different compartments of the lungs.

The authors interpret their findings as a further indication that the antioxidant defences in smokers are impaired, which puts them at increased risk for the noxious effects of oxidative stress. TAGER *et al.* [43] reported evidence for a reduced cellular GSH expression in alveolar macrophages of chronic smokers and COPD patients. Beyond this, macrophages in the BAL of smokers are phenotypically heterogeneous, partially altered in size, full with engulfed particles or immature [44]. The current findings suggest that the intracellular GSH deficiency of BAL cells is the result of a functional disturbance in the GSH *de novo* synthesis due to a decreased expression of the GCLM. These data provide additional support for the view that alveolar macrophages are highly compromised by oxidative stress induced by cigarette smoke *in vivo*. The regulatory mechanisms that maintain the ELF GSH content *in vivo* are still unresolved, but based on this study, GCL expression in alveolar macrophages may not be responsible for the high ELF GSH content in smokers.

Studies are underway to confirm these findings and to identify the specific cells and mechanisms that underlie the upregulation of glutathione in the epithelial lining fluid of smokers.

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