Original Contribution

LOCALIZATION OF \( \gamma \)-GLUTAMYLCYSTEINE SYNTHETASE MESSENGER RNA EXPRESSION IN LUNGS OF SMOKERS AND PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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Abstract—Cigarette smoking results in an oxidant/antioxidant imbalance in the lungs and inflammation, which are considered to be key factors in the pathogenesis of chronic obstructive pulmonary disease (COPD). Glutathione (GSH) is an important protective antioxidant in lung epithelial cells and epithelial lining fluid. De novo GSH synthesis in cells occurs by a two-enzyme process. The rate-limiting enzyme is \( \gamma \)-glutamylcysteine synthetase (\( \gamma \)-GCS), in which the heavy subunit (HS) constitutes most of its catalytic activity. The localization and expression of \( \gamma \)-GCS-HS in specific lung cells as well as possible differences in its expression between smokers with and without COPD have not yet been studied. The purpose of this study was to investigate \( \gamma \)-GCS-HS expression using messenger RNA in situ hybridization in peripheral lung tissue. We studied 23 current or ex-smokers with similar smoking histories with \( n = 11 \); forced expiratory volume in 1 s [FEV\(_1\)] < 75% predicted) or without COPD \( n = 12 \); FEV\(_1\) > 84% predicted). We assessed the relations between pulmonary \( \gamma \)-GCS-HS expression, FEV\(_1\) and transforming growth factor-\( \beta \) (TGF\( \beta \)), because TGF\( \beta \) can modulate \( \gamma \)-GCS-HS expression in lung epithelial cells. Gamma-GCS-HS is predominantly expressed by airway and alveolar epithelial cells, alveolar CD68\(^+\) cells (macrophages), and endothelial cells of both arteries and veins. In subjects with COPD, semiquantitative analysis revealed higher levels of \( \gamma \)-GCS-HS messenger RNA in alveolar epithelium (1.5 times, \( p = .04 \)) and a trend for a higher expression in bronchiolar epithelium (1.3 times, \( p = .075 \)) compared with subjects without COPD. We did not observe a significant correlation between airway and alveolar epithelial \( \gamma \)-GCS-HS expression and TGF\( \beta \) expression \( r = .20 \), FEV\(_1\) percentage predicted \( r = .18 \), or FEV\(_1\)/forced vital capacity ratio \( r = .14 \); \( p > .05 \). Our results show that \( \gamma \)-GCS-HS is localized, particularly in lung epithelium, and shows higher expression in smokers with COPD. This suggests a specific role for enhanced GSH synthesis as a mechanism to provide an adaptive response against oxidative stress in patients with COPD. © 2000 Elsevier Science Inc.

Keywords—Glutathione, \( \gamma \)-Glutamylcysteine synthetase, mRNA, Smokers, COPD, Lungs, Free radical

INTRODUCTION

The tripeptide, \( L-\gamma \)-glutamyl-L-cysteinylglycine, or glutathione (GSH), is an ubiquitous nonprotein sulfhydryl compound that has an important role in maintaining intracellular redox balance and cellular defenses against oxidative stress [1,2]. Depletion of cellular GSH is associated with lung damage produced by a variety of oxidants [1,3]. GSH is present in high concentrations in the lung epithelial lining fluid (ELF) [4]. It is also important in maintaining the integrity of the airspace epithelium in type II alveolar cells in vitro and in lungs in vivo [3,5].

GSH is synthesized by two enzymes, \( \gamma \)-glutamylcysteine synthetase (\( \gamma \)-GCS), which is the rate-limiting enzyme, and glutathione synthetase [6]. The \( \gamma \)-GCS holoenzyme exists as a dimer composed of a heavy subunit (\( \gamma \)-GCS-HS) and a light subunit (\( \gamma \)-GCS-LS) [7]. The heavy subunit possesses all the catalytic activity [8]. We [11–15] as well as other investigators [9,10] have re-
recently shown that the γ-GCS-HS gene is induced in response to variety of agents such as oxidants, phenolic antioxidants, and tumor necrosis factor-α (TNF-α) in alveolar epithelial cells.

A physiological role for GSH as an antioxidant has been described in numerous inflammatory disorders [16]. Chronic obstructive pulmonary disease (COPD) is a condition characterized by airspace inflammation and progressive and largely irreversible airway obstruction [17]. One of the important events in the pathogenesis of COPD is considered to be the creation of an imbalance between oxidants and antioxidants in the lungs [17]. Cigarette smoke, which contains an estimated 10^{14} free radicals per puff, is the major etiological factor in the pathogenesis of COPD [18]. The response of antioxidants such as GSH in response to smoking may be a factor in susceptibility to the development of COPD. We have recently shown that cigarette smoke condensate, oxidants, and TNF-α impose oxidative stress, resulting in transient depletion of intracellular GSH followed by rapid induction of glutathione synthesis in alveolar epithelial cells (A549) in vitro [11-15]. These data are supported by studies showing elevated levels of GSH in ELF in chronic smokers compared with nonsmokers [19,20]. Preliminary data showed increased expression of γ-GCS-HS messenger RNA (mRNA) in bronchial biopsies in chronic smokers in contrast to low levels of GSH in ELF and decreased γ-GCS-HS mRNA expression in bronchial biopsies after acute smoking [20,21]. The site of GSH synthesis in the different cell types in the lungs is unknown, however, its regulation in the lungs of smokers and in patients with COPD has not been studied.

In situ hybridization allows the identification of cells expressing γ-GCS-HS mRNA sequences directly on tissue sections and, hence, the precise localization of γ-GCS-HS mRNA expression. In this study, we used in situ hybridization to investigate the expression and localization of γ-GCS-HS mRNA in lung cells and to study differences in the expression of γ-GCS-HS mRNA in lung tissue of smokers with and without COPD. Previously, we have shown an increase in transforming growth factor-β1 (TGF-β1) expression in bronchial and alveolar epithelium in subjects with COPD [22]. TGF-β1 has been shown to modulate GSH synthesis in lung cells in vitro [23]. We hypothesized that the increased expression of TGF-β1 in lungs of COPD patients may be one factor that alters GSH synthesis by its regulatory effect on γ-GCS-HS mRNA. We therefore examined the relation between γ-GCS-HS mRNA expression and TGF-β1 as well as the relation with measurements of airflow obstruction in patients with COPD.

### MATERIALS AND METHODS

#### Subjects

In this study, we used the same lung tissue specimens from current or exsmokers with or without COPD who were undergoing lung resection for lung cancer as described previously [24]. We selected tissue specimens containing peripheral airways (airway diameter ranging from 1 to 3 mm). Eleven subjects with COPD with a forced expiratory volume in 1 s (FEV₁) less than 75% of the predicted value before bronchodilation (FEV₁/forced vital capacity [FVC] ratio < 70% predicted; 7 exsmokers and 4 current smokers) and 12 subjects without COPD (FEV₁ > 84% predicted; 8 exsmokers and 4 current smokers) were studied. The smoking history of subjects with and without COPD was similar (p > .05) (Table 1). All subjects showed no reversibility in FEV₁ of less than 13% after 400 μg of inhaled salbutamol. Exclusion criteria included: (i) diffuse pulmonary inflammation associated with lung fibrosis, (ii) absence of tumorfree and pneumonia free lung tissue specimens, and (iii) obstruction of central bronchi as a result of the tumor [24]. Preoperatively, none of the patients had clinical evidence of an upper respiratory tract infection, and none had received antibiotics in the 4 weeks before surgery or glucocorticosteroids during the 3 months before surgery, with the exception of 3 patients who received glucocorticosteroids only perioperatively (Table 1). Data on lung function tests of these patients are presented in Table 1.

**γ-GCS-HS digoxigenin riboprobe synthesis**

The 1.679 kilobase complementary DNA insert of human γ-GCS-HS in pBluescript II SK (ATCC 79023) (Stratagene, La Jolla, CA, USA) [25] was used to derive the complementary RNA (cRNA) sense and antisense
probes. The whole plasmid containing γ-GCS-HS complementary DNA was digested using Pst I, resulting in a 760 base pair fragment, and was recloned into pBlue-Script II SK. The plasmid was linearized by restriction digestion using Bam H1 and Hind III (Promega, Southampton, UK) (Fig. 1). T7 and T3 RNA polymerases (Boehringer Mannheim, Mannheim, Germany) were used to make both antisense and sense probes, respectively. Digoxigenin-labeled cRNA probes for γ-GCS-HS were synthesized using digoxigenin-11-UTP and procedures provided by the manufacturer (Boehringer Mannheim).

In situ hybridization

Tissue sections (3 μm thick) were deparaffinized, rehydrated, and pretreated before in situ hybridization. The mRNA in situ hybridization was performed essentially as described previously [22,26]. Briefly, after pretreatment, the sections were hybridized with 100 ng per slide for 16 h at 62°C. Subsequently, sections were washed in 2× standard saline citrate (SSC) with 50% formamide at 50°C, washed in 0.1× SSC with 20 mM of β-mercaptoethanol at 62°C, and finally treated with 2 U/ml of ribonuclease T1 (Boehringer Mannheim) in 2× SSC plus 1 mM of ethylenediaminetetraacetic acid at 37°C. The immunohistochemical detection of digoxigenin-labeled hybrids was performed using a sheep antibody against digoxigenin (Boehringer Mannheim). For the color development, we used nitroblue tetrazolium as a chromogen and bicholyl-indolyl phosphate as a coupling agent (Boehringer Mannheim). The sense ribo-probes were included as negative controls and did not show staining. The staining intensity was assessed semiquantitatively [22] in a blinded fashion by a pathologist using an arbitrary scale (range: 0, no staining; 1, moderate staining; 2, intense staining; 3, very intense staining).

Statistical analysis

In situ hybridization data were expressed as mean ± SEM. For all data, the distribution was Gaussian. Significance levels for γ-GCS expression were obtained using an unpaired, two-tailed, Student’s t-test, with Welch’s correction if variances were different. Correlation of the γ-GCS expression with FEV1 percentage predicted or TGFβ1 expression was done using the Pearson correlation test. At a probability value of less than .05, differences were considered to be statistically significant.

RESULTS

In all subjects, γ-GCS-HS mRNA was localized predominantly in bronchial, bronchiolar, and alveolar epithelial cells; endothelial cells; and CD68+ cells (which are considered to be macrophages) (Fig. 2). Subepithelial cells, including inflammatory cells like lymphocytes, were less intensely stained. Smooth muscle cells rarely stained for γ-GCS-HS.

In subjects with COPD, we observed significantly higher (1.5 times, \( p < .04 \) with Welch’s correction) γ-GCS-HS mRNA levels in the alveolar epithelium, with a similar trend in airway epithelial cells (1.3 times, \( p = .075 \)) than in subjects without COPD (Fig. 3). The lack of greater differences in γ-GCS-HS expression may possibly be a result of the relative homogeneity of the population studied, which did not include healthy nonsmokers.

Recently, we showed (in the same subject groups used in this study) enhanced levels of TGF-β1 in bronchiolar and alveolar epithelium in patients with COPD [22]. Because TGF-β1 was reported to downregulate γ-GCS-HS gene transcription [23], we examined the relation between γ-GCS-HS mRNA and TGF-β1 mRNA levels in airway and alveolar epithelium. We did not find any significant correlations between γ-GCS-HS mRNA and TGF-β1 mRNA levels in airway or alveolar epithelium and TGF-β1 mRNA levels in airway and alveolar epithelium. We did not find any significant correlations between γ-GCS-HS mRNA in airway or alveolar epithelium and TGF-β1 mRNA and protein levels (\( r = .20 \), or between FEV1 \( r = .18 \) or FEV1/FVC \( r = .14 \) in subjects without or with COPD \( p > .05 \). Hence, alveolar epithelial γ-GCS-HS mRNA and TGF-β1 mRNA and protein levels are independent factors associated with COPD.
DISCUSSION

Previously, it has been shown that γ-GCS-HS mRNA is expressed in human lungs using Northern blot analysis [27]. In addition, high levels of GSH have been demonstrated in resected lung tumor tissue [28,29]. In this study, using digoxigenin-labeled γ-GCS-HS cRNA probes, we show that γ-GCS-HS mRNA expression occurs in bronchiolar and alveolar epithelium, in interstitial and intraluminal CD68+ cells (macrophages), and in endothelium in resected peripheral lung tissue of subjects with and without COPD. In subjects with COPD, a higher expression of γ-GCS-HS mRNA was seen in airways and alveolar epithelial cells with no difference in alveolar macrophages than in subjects without COPD.

Alveolar epithelial type II cells are more resistant to oxidant stress than other lung cells [30]. The reason for this difference may be the presence of increased levels of antioxidant enzymes, particularly GSH and its redox system. Hence, intracellular GSH is likely to be important in the protection against oxidant-induced alveolar injury. Rapid induction of GSH synthesis occurs in response to various oxidant stresses [9,10,14,15,31] and is therefore likely to be a critical determinant of cellular tolerance.

The levels of GSH and γ-GCS activity are regulated by the expression of γ-GCS-HS [8,9]. Recently, we have shown that oxidants and cigarette smoke can increase GSH concentrations by induction of γ-GCS-HS expression in A549 alveolar epithelial cells [12–14]. Thus, our data demonstrating an increased expression of γ-GCS-HS mRNA in the alveolar epithelium of smokers with COPD...
COPD suggest an adaptive response to oxidative stress in the lungs of these patients.

TGF-β1 is a multifunctional growth factor that modulates cellular proliferation, differentiation, and tissue repair [32]. In smokers without COPD, TGF-β1 protein and mRNA expression have been shown in bronchial and alveolar epithelial cells, alveolar macrophages, and smooth muscle cells [33]. We have recently shown that TGF-β1 is localized mainly to bronchiolar and alveolar epithelium and macrophages and that epithelial TGF-β1 expression is higher in smokers with COPD than in smokers without COPD [22]. In addition, Vignola and coworkers [34] reported higher epithelial TGF-β1 expression in lung tissue from smokers with chronic bronchitis than in nonsmokers. TGF-β1 has been shown to act as a pro-oxidant molecule in endothelial cells by increasing the cellular release of hydrogen peroxide [35]. TGF/β1 also induces a marked decrease in endothelial and alveolar epithelial cell GSH and downregulates γ-GCS-HS mRNA levels in vitro [23,36,37]. The two effects do not occur simultaneously, however, and seem to be independent of each other [36,37]. In this study, we were unable to establish a relation between γ-GCS-HS mRNA expression and levels of TGF-β1 in smokers with or without COPD. This may be a result of other confounding factors such as differences in local TNF-α levels, which have been shown to be higher in patients with COPD [38]. TNF-α is known to upregulate γ-GCS-HS mRNA in lung epithelial cells [15]. It is also possible that γ-GCS-LS might be influenced by TGF-β1 expression, which requires further study. The induction of γ-GCS-LS by cytokines and growth factors has not been studied to date. Thus, the balance/equilibrium between gene expression of proinflammatory mediators, growth factors, and protective antioxidants in the inflammatory processes in the lungs of patients with COPD is currently not known. Furthermore, we also did not find any significant relation between γ-GCS-HS expression and FEV1 in smokers with or without airway obstruction. Linden et al. [39] showed a relation between bronchoalveolar lavage fluid (BALF) GSH concentrations and FEV1 in smokers with and without chronic bronchitis (i.e., the higher the BALF GSH, the lower the FEV1). The discordance between our data and those of Linden’s group may be explained by the fact that BALF GSH concentrations and γ-GCS-HS expression may be influenced by the recent smoking history, because chronic smoking leads to increased levels of γ-GCS-HS mRNA in bronchial biopsies compared with those of nonsmokers [21]. The lack of a correlation between γ-GCS-HS expression and measurements of airflow obstruction may be a result of the small numbers in this study.

In conclusion, this study showed that in smokers with and without COPD, γ-GCS-HS mRNA expression occurs in airway epithelial cells, endothelial cells, and macrophages. Higher γ-GCS-HS expression in alveolar epithelial cells occurred in smokers or exsmokers with COPD than in smokers without COPD. These findings support the concept that elevated GSH and γ-GCS-HS mRNA levels found in ELF and bronchial biopsies respectively in smokers. This study suggests that γ-GCS-HS may provide an adaptive mechanism in response to oxidant stress and cigarette smoke–induced oxidative stress. This adaptation may be more pronounced in those who develop COPD.

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ABBREVIATIONS

BALF—bronchoalveolar lavage fluid
cRNA—complementary RNA
COPD—chronic obstructive pulmonary disease
ELF—epithelial lining fluid
FEV1—forced expiratory volume in 1 s
FVC—forced vital capacity
GSH—glutathione
γ-GCS—HS—γ-glutamylcysteine synthetase—heavy subunit
γ-GCS—LS—γ-glutamylcysteine synthetase—light subunit
mRNA—messenger RNA
SSC—standard saline citrate
TGFβ1—transforming growth factor-β1
TNFα—tumor necrosis factor-α