Cancer-associated differences in acetylcholinesterase activity in bronchial aspirates from patients with lung cancer

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ABSTRACT

In non-neuronal contexts, ACh (acetylcholine) is thought to be involved in the regulation of vital cell functions, such as proliferation, differentiation, apoptosis and cell–cell interaction. In airways, most cells express the non-neuronal cholinergic system, each containing a specific set of components required for synthesis, signal transduction and ACh hydrolysis. The aim of the present study was determine the expression of cholinergic system components in bronchial aspirates from control subjects and patients with lung cancer. We conducted an analysis of cholinergic components in the stored soluble and cellular fraction of bronchial aspirates from non-cancerous patients and patients diagnosed with lung cancer. The results show that the fluid secreted by human lung cells contains enough AChE (acetylcholinesterase) activity to control ACh levels. Thus these findings demonstrate that: (i) AChE activity is significantly lower in aspirates from squamous cell carcinomas; (ii) the molecular distribution of AChE in both bronchial cells and fluids consisted of amphiphilic monomers and dimers; and (iii) choline acetyltransferase, nicotinic receptors and cholinesterases are expressed in cultured human lung cells, as demonstrated by RT–PCR (reverse transcriptase–PCR). It appears that the non-neuronal cholinergic system is involved in lung physiology and lung cancer. The physiological consequences of the presence of non-neuronal ACh will depend on the particular cholinergic signalling network in each cell type. Clarifying the pathophysiological actions of ACh remains an essential task and warrants further investigation.

Key words: acetylcholinesterase, airway surface fluid, cancer, lung, proliferation.

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AChE-R, stress-induced AChE variant; AC, adenocarcinoma; ASF, airway surface fluid; BChE, butyrylcholinesterase; ChAT, choline acetyltransferase; HRP, horseradish peroxidase; iso-OMPA, tetraisopropyl pyrophosphoramide; mACHR, muscarinic ACh receptor; nACHR, nicotinic ACh receptor; RT–PCR, reverse transcriptase–PCR; SCC, squamous cell carcinoma; SCLC, small-cell lung carcinoma; NSCLC, non-SCLC; TBS, Tris-buffered saline.

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INTRODUCTION

As a neurotransmitter, ACh (acetylcholine) plays pivotal roles in both the central and peripheral nervous systems. ACh is also a signalling molecule in the lung (for reviews see [1,2], and also below). Thus ACh plays two different biological roles, acting as a classical neurotransmitter (neuronal) and as a cytomolecule (non-neuronal). The presence of cholinergic components in epithelial cells (intestine, skin and urogenital tract), and mesothelial, endothelial, muscle and immune cell tissues points to the existence of a cholinergic phenotype, modulating basic cell functions, such as gene expression, proliferation, differentiation, cellular adhesion, migration, secretion and absorption.

Multiple studies have established that human lung cells express the non-neuronal cholinergic system (reviewed in [3]), i.e. the synthesizing enzyme ChAT (choline acetyltransferase) [3–5], nAChR and mAChR (nicotinic and muscarinic ACh receptors respectively) [6–11], as well as enzymes for ACh hydrolysis AChE (acetylcholinesterase) and BChE (butyrylcholinesterase) [12,13].

In the lung, the role of ACh as an autocrine/paracrine signalling molecule is not yet generally accepted, despite many findings showing that lung cells synthesize the cholinergic components [14]. Importantly, the case for non-neuronal autocrine signalling has also been reported in lung cancers [6,15,16]. The fact that nicotine regulates cellular proliferation by activation of Akt [17] or by β-arrestin-mediated activation of Src and Rb (retinoblastoma)/Raf-1 pathways [18], at the same time as blocking apoptosis [16,19], provides strong evidence that stimulation of nAChR and/or mAChR by endogenous ACh or exogenous agonists associated with tobacco consumption could participate actively in the pathogenesis of lung cancer. The biochemical events between ligands and receptors leading to carcinogenesis and tumour progression are not fully understood. Another point of growing interest links the cholinergic system and angiogenic activity in tumours. Nicotine and endogenous cholinergic pathways could mediate endothelial cell growth and angiogenesis [20–22]. Increasing evidence has shown that AChE may be involved in pivotal processes of carcinogenesis and tumour progression. Thus, in leukaemia, ovarian carcinoma and tumour cell lines, aberrations in the AChE gene are frequently found (reviewed [23–25]). A role of AChE expression during apoptosis has been demonstrated [26,27], and the mechanism underlying AChE up-regulation is mediated by the JNK (c-Jun N-terminal kinase)/c-Jun pathway [28]. Recently, we have shown that post-translational processing of AChE is altered in lung cancer in a cell-type-dependent manner [13].

The aim of the present study was to determine the level of AChE activity, to investigate the pattern of molecular forms and to detect the expression of cholinergic compo-

nents important in cholinergic signalling. We show that AChE activity was found in bronchial aspirates, and that the differences between lung diseases may have potential diagnostic uses.

MATERIAL AND METHODS

Chemicals

Acetylthiocholine iodide, DTNB ([5,5′-dithiobis-(2-nitrobenzoic acid)], iso-OMPA (tetrakisopropyl pyrophosphoramide), Brij 96, enzyme markers for sedimentation analysis (bovine liver catalase (11.4 S20,w) and bovine intestine alkaline phosphatase (6.1 S20,w)), HRP (horseradish peroxidase)-conjugated monoclonal anti-(goat IgG) antibodies and recombinant human AChE were purchased from Sigma. Goat antiserum against the N-terminal peptide of human AChE (N19) was purchased from Santa Cruz Biotechnology. Molecular-mass protein standards were from Invitrogen. For RT–PCR (reverse transcriptase–PCR) assays, mRNA was extracted using a Chemagic mRNA Direct kit (Chemagen). The GenAmp RNA PCR kit (Applied Biosystems) was used for reverse transcription. Other chemicals were of analytical grade.

Patients

We studied 105 patients admitted to the Virgen de la Arrixaca University Hospital in Murcia between 2003 and 2006; in all of whom pulmonary function was affected. Haemolytic bronchial aspirates (n = 20) were discarded. A total of 85 samples of bronchoalveolar aspirates from patients without (control; n = 49) or with (n = 36) lung cancer were studied. Of these, 70 were male and 15 were female, and their ages ranged from 20 to 88 years. The mean age was 62.60 years.

Histological evaluation of the tumours, according to WHO (World Health Organization) criteria, revealed that ten corresponded to AC (adenocarcinoma), 15 to SCC (squamous cell carcinoma) and 11 to SCLC (small-cell lung carcinoma). Staging was assessed according to the TNM classification system (Tumor Node Metastasis Staging System).

All patients gave their consent after being appropriately informed, and the Ethics Committee of the University Hospital Virgen de la Arrixaca reviewed and approved the study.

Cell culture

The human NSCLC (non-SCLC) cell lines NCI-H1264, NCI-H157, A549 and H23, and the human SCLC cell lines DMS-79, NCI-H169, NCI-H187 and N-417 were gifts from Dr L. Montuenga (Department of Histology and Pathology, Schools of Sciences and Medicine, University of Navarra, Navarra, Spain) and Dr M. Sanchez-Cespedes (Lung Cancer Group, Centro Nacional de Investigaciones Oncologicas, Madrid,
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The Caco-2 colon cancer cell line was a gift from the Experimental Science Support Service (University of Murcia, Murcia, Spain). The human breast cancer cell line MCF-7 and human melanoma SK-MEL-28 were purchased from the A.T.C.C. Cell lines were maintained in RPMI medium supplemented with 10% (v/v) fetal bovine serum, 2 mmol/l l-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in an atmosphere of 5% CO₂.

Processing of the samples
Bronchial aspirates were performed by staff physicians in a standard fashion using a 8.0-mm fibre-optic bronchoscope. Subjects were given local anaesthesia using 4% lidocaine sprayed in the oropharynx and 1% lidocaine instilled over the vocal cords, carina and bronchus. The bronchial aspirates were centrifuged at 462 g for 10 min at 4 °C to pellet the cells. All fractions were rapidly frozen at −80 °C until required. Serum from patients was obtained by drawing whole blood using standard procedures on the same day before the collection of bronchial aspirates.

Cytological examination of the cellular fraction of bronchial aspirates was performed by the pathologists and only cases with unequivocal malignant features allowing tumour typing were considered. Cellular fractions of bronchial aspirates were found to contain ciliated cells in a much greater proportion than squamous cells and macrophages. Both the number of recovered cells and the percentage of viable cells (as estimated by Trypan Blue staining) varied significantly between samples. The number and percentage of cancerous cells in bronchial aspirates from patients with lung cancer ranged from a few cells to several thousand.

Assay of the AChE activity in bronchial aspirates
Recovered bronchial aspirates are variable mixtures of sterile saline solution, ASF (airway surface fluid) and ASF components. Therefore the concentration of recovered molecules in ASF is usually very low because of the saline solution added. Measurement of AChE activity in such samples required a very sensitive methodology. The Ellman method [29] is not sensitive enough to measure AChE activity in such diluted samples. Therefore AChE activity was determined using the ACh/AChE assay kit (Molecular Probes), according to the manufacturer’s instructions. AChE activity in bronchial aspirate fractions was measured by adding 0.1 ml of serially diluted samples in duplicate into 96-well microplates. Subsequently, 0.1 ml of the mixture reaction [50 mmol/l Tris/HCl (pH 7.5), 0.2 mmol/l Amplex Red reagent, 2 units/ml HRP and 0.2 unit/ml choline oxidase] was added. Reactions were measured using an excitation wavelength of 530 nm and an emission wavelength of 590 nm for 30 min at multiple time points to follow the enzyme kinetics. A standard curve [0–20 m-units/ml of AChE from electric eel (included with the assay kit)] was used in each experiment. One unit of AChE activity represents the hydrolysis of 1 μmol of substrate/min at 37 °C.

Standardization of AChE activity in bronchial aspirates with urea
It is difficult to estimate the actual concentration of recovered molecules in ASF in situ because, when the saline solution is recovered by aspiration, ASF and its components are recovered along with it. The degree to which the process of collecting bronchial aspirates dilutes the ASF was determined using urea as a marker of dilution [30]. Serum and ASF urea concentrations were determined using the kinetic UV assay for urea (Roche Diagnostic), according to the manufacturer’s instructions, in a microplate spectrophotometer (Biotek Instrument) and using a standard curve (0–20 mg/dl urea). The following formula was used:

\[
\text{Concentration in ASF} = \frac{\text{concentration in samples} \times \text{serum urea concentration}}{\text{sample urea concentration}}.
\]

Solubilization of AChE from lung epithelial and cultured cells
AChE was extracted from the airways cells by homogenization in 0.5 ml of TBS [Tris-buffered saline; 10 mmol/l Tris/HCl (pH 7.5), 1 mol/l NaCl and 50 mmol/l MgCl₂] supplemented with 0.25% Brij 96. After centrifugation at 37 500 g for 30 min at 4 °C in a TLA rotor (Beckman), the supernatant containing the soluble and membrane-bound AChE molecules was recovered and stored at −80 °C until use. AChE activity in supernatants was measured by the Ellman method [29]. AChE was assayed with 1 mmol/l acetylthiocholine and 100 μmol/l iso-OMPA (an inhibitor of BChE), and 1 unit of AChE activity represents the hydrolysis of 1 μmol of substrate/min at 37 °C. The hydrolysis of substrate due to non-specific esterases was negligible.

The amount of protein was determined by the Bradford method [31] with BSA as the standard.

Sedimentation analysis
The molecular distribution of AChE in both bronchial cells and fluids was studied by sedimentation analysis in sucrose continuous gradients, as described previously [13]. Briefly, samples and sedimentation markers (bovine liver catalase and intestine alkaline phosphatase) were layered on the top of centrifuge tubes containing 5–20% sucrose gradients, in the presence of 0.5% Brij 96 detergent. The gradient tubes were centrifuged at 35 000 rev./min for 18 h at 4 °C in a SW41Ti rotor (Beckman). After centrifugation, fractions of 250 μl
were collected from the bottom of the tube and assayed for AChE activity and enzyme markers to obtain the sedimentation profiles.

**RT–PCR**

The expression of the components of the cholinergic system, such as ChAT, the enzyme that synthesizes ACh, AChE, the enzyme that hydrolyses it, and nAChR subunits, was studied by RT–PCR. mRNA was extracted from cultured human tumour cells using the Chemagic mRNA Direct kit (Chemagen) and reversed-transcribed into cDNA by random priming using the GeneAmp RNA PCR kit (Applied Biosystems). Subsequently, 2–5 μl of cDNA was amplified by 40 cycles of PCR (10 s at 95 °C, 15 s at 55–60 °C, 15 s at 72 °C) on a LightCycler (Roche) using the primer pairs listed in Table 1. Negative controls (without reverse transcriptase) for each primer pair were assayed. The primer pairs employed to detect the mRNA of the cholinergic components are listed in Table 1, where the strategy (intron-spanning/intron-flanking) employed is indicated. β-Actin mRNA was used as an internal control. The PCR products were visualized by electrophoresis on 1.5 % (w/v) agarose gels.

**Western blotting**

AChE subunits from both ASF and bronchial cells were resolved by reductive SDS/PAGE [32] on 12.5 % (w/v) polyacrylamide gels. Proteins were electrotransferred on to PVDF membranes. The membranes were blocked with 5 % (w/v) non-fat dried milk in TBS containing 0.1 % Tween 20 and then incubated with the anti-AChE antisera (N19; Santa Cruz Biotechnology). As N19 antibodies are produced against the N-terminal peptide of human AChE, it should react with the full set of AChE variants. No bands were obtained in negative control samples (without primary antibody N19) thus verifying that the protein bands corresponded with proteins specifically recognized by the primary antibody. Labelled proteins were revealed with HRP-conjugated anti-(goat IgG) antibodies and the ECL® Western blotting detection system (GE Healthcare). Blots were exposed to Hyperfilm-ECL® (GE Healthcare) for various periods of time. The molecular mass of the AChE subunits was estimated using appropriate protein standards (ECL® DualVue; GE Healthcare), and the intensity of the protein bands was quantified using GelPro Analyzer Software (version 3.1; Media Cybernetic). β-Actin was used as a loading control, and recombinant AChE (1, 5 or 10 μg) was used as a positive control (see Figure 2).

**Statistical analysis**

The results are given as means ± S.E.M. The statistical significance of the difference in ACh content and AChE activity between the cancerous and non-cancerous groups was evaluated using a Student’s t test. The non-parametric Mann–Whitney U test (signed rank test at

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used for RT–PCR</th>
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<tr>
<td>Gene</td>
<td>Primer</td>
</tr>
<tr>
<td>AChE variant T</td>
<td>Forward 5′ ACTTGTCCGCGCAGGGGA 203*</td>
</tr>
<tr>
<td>AChE variant T</td>
<td>Reverse 5′ GCCCTGGGACGGCTGGTTT</td>
</tr>
<tr>
<td>AChE variant H</td>
<td>Forward 5′ ACTTGTCCGCGCAGGGGA 201*</td>
</tr>
<tr>
<td>AChE variant H</td>
<td>Reverse 5′ GGGACCTCGAGGGCTGGT</td>
</tr>
<tr>
<td>AChE variant R</td>
<td>Forward 5′ CCCCCGACCCCCCCGAAAC 315†</td>
</tr>
<tr>
<td>AChE variant R</td>
<td>Reverse 5′ ACCTGCGGGGCTCCACTC</td>
</tr>
<tr>
<td>AChE E1d</td>
<td>Forward 5′ CTTGATGCAGGCCACCCAGA</td>
</tr>
<tr>
<td>AChE E1d</td>
<td>Reverse 5′ TCTCCAGCCCAGGGGCGAGG</td>
</tr>
<tr>
<td>BChE</td>
<td>Forward 5′ GTGTGGTCTACCCGCGAAG 297†</td>
</tr>
<tr>
<td>BChE</td>
<td>Reverse 5′ CACTCCCCATTCTGCTCAC</td>
</tr>
<tr>
<td>ChAT</td>
<td>Forward 5′ TACGCTAGCTGCGGACA</td>
</tr>
<tr>
<td>ChAT</td>
<td>Reverse 5′ ATAGGCTTTCTCACCAGC</td>
</tr>
<tr>
<td>nAChR α4</td>
<td>Forward 5′ CAGTCTCAGCGCTGACCT 283†</td>
</tr>
<tr>
<td>nAChR α4</td>
<td>Reverse 5′ AGCCGATGTCAGCGTTC</td>
</tr>
<tr>
<td>nAChR α7</td>
<td>Forward 5′ GAGGCCAAGAAGATCGATG 114*</td>
</tr>
<tr>
<td>nAChR α7</td>
<td>Reverse 5′ TTGAGAAACTTTCTCCTGTG</td>
</tr>
<tr>
<td>nAChR α3</td>
<td>Forward 5′ GACCGAGCTGCGTGCAGG 110*</td>
</tr>
<tr>
<td>nAChR α3</td>
<td>Reverse 5′ AGAGGGAGACCCACACATT</td>
</tr>
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*Intron-spanning; †Intron-flanking. AChE E1d, N-terminus-extended AChE.

P < 0.05) was used to compare AChE between the several histological types of lung cancer. Statistical analysis was performed using the SPSS software program (version 10 for Windows; SPSS).

**RESULTS**

**AChE activity in ASF from patients without and with cancer**

ASF obtained from the patients contained AChE activity. The AChE activity in non-cancerous samples was 1.34 ± 0.17 m-units/ml and decreased to 1.18 ± 0.18 m-units/ml in tumoral bronchial aspirates (Table 2A). Significantly different levels of AChE activity were detected in bronchial aspirates from patients diagnosed with SCLC, AC and SCC (Table 2B). We have reported previously that AChE activity varied according to the histological classification of lung tumours [12]. In accordance with these previous findings, AChE activity in ASF from
Table 2  AChE activity in ASF of bronchial aspirates
Values are means ± S.E.M., with medians (first–third quartile). ∗The statistical difference between AChE activity in non-cancerous and cancerous samples was evaluated by Student’s t test. The non-parametric Mann–Witney U test was used to determine statistical significance (P < 0.05) according to histology of lung cancer. COPD, chronic obstructive pulmonary disease.

<table>
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<tr>
<th>Diagnosis</th>
<th>AChE (m-units/ml)</th>
<th>n</th>
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<tbody>
<tr>
<td>Non-cancerous</td>
<td>1.34 ± 0.17 [0.542 (1.111–2.082)]</td>
<td>37</td>
</tr>
<tr>
<td>Cancerous</td>
<td>1.18 ± 0.18 [0.249 (0.853–1.807)]</td>
<td>35</td>
</tr>
<tr>
<td>AC</td>
<td>2.17 ± 0.44 [1.270 (2.626–3.013)]</td>
<td>7</td>
</tr>
<tr>
<td>SCC</td>
<td>0.51 ± 0.23 [0.274 (0.225–0.662)]</td>
<td>10</td>
</tr>
<tr>
<td>SCLC</td>
<td>0.91 ± 0.24 [0.270 (0.876–1.485)]</td>
<td>7</td>
</tr>
<tr>
<td>COPD</td>
<td>0.93 ± 0.47 [0.201 (0.529–1.613)]</td>
<td>6</td>
</tr>
<tr>
<td>Bronchitis</td>
<td>0.56 ± 0.13 [0.225 (0.392–0.898)]</td>
<td>9</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>0.66 ± 0.27 [0.191 (0.558–1.222)]</td>
<td>4</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.92 ± 0.18 [0.175 (0.629–1.490)]</td>
<td>25</td>
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Comparison  | P value       |
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<tr>
<td>Non-cancerous compared with cancerous</td>
<td>0.434∗</td>
</tr>
<tr>
<td>Non-cancerous compared with AC</td>
<td>0.038</td>
</tr>
<tr>
<td>Non-cancerous compared with SCC</td>
<td>0.007</td>
</tr>
<tr>
<td>Non-cancerous compared with SCLC</td>
<td>0.431</td>
</tr>
<tr>
<td>AC compared with SCC</td>
<td>0.012</td>
</tr>
<tr>
<td>AC compared with SCLC</td>
<td>0.053</td>
</tr>
<tr>
<td>SCC compared with SCLC</td>
<td>0.133</td>
</tr>
<tr>
<td>COPD compared with non-COPD</td>
<td>0.616</td>
</tr>
<tr>
<td>Bronchitis compared with non-bronchitis</td>
<td>0.143</td>
</tr>
<tr>
<td>Pneumonia compared with non-pneumonia</td>
<td>0.607</td>
</tr>
<tr>
<td>Smoking compared with non-smoking</td>
<td>0.924</td>
</tr>
</tbody>
</table>

patients with SCC were greatly reduced compared with the activity in patients with AC. No significant differences were observed in the AChE activity of ASF from patients with a smoking habit, COPD (chronic obstructive pulmonary disease), bronchitis or pneumonia.

AChE molecules in bronchial aspirates
The pattern of molecular forms of AChE in both soluble and cellular fractions of bronchial aspirates was investigated by sedimentation analysis (Figure 1). Abundant 4.4 ± 0.2 S and less 5.9 ± 0.2 S and 2.8 ± 0.1 S AChE forms were identified (Figure 1). These molecular forms corresponded to dimeric (G₂A; 4.5 S) and monomeric (G₁A; 2.8 S) amphiphilic AChE molecules, whereas the 5.9 S molecules were assigned to hydrophilic dimers of AChE (G₂H). All of the samples included in the present study had a similar pattern of AChE forms, indicating that, whatever the pulmonary diseases are, they do not affect the biosynthesis and/or secretion of AChE molecules. Sedimentation analysis of cellular extracts from cultured cancer cells of epithelial origin, such as lung (A549, H157, N417 and H69 cells), breast (MCF-7 cells) and colon (Caco-2 cells), had almost identical AChE molecular compositions. Tetrameric globular forms (G₄)
Figure 2  Immunoblotting of AChE protein in ASF
Proteins (50 μg) in non-tumoral (NT), AC (A), SCC (S) and SCLC ASF were resolved by reductive SDS/PAGE, as described in the Material and methods section. N19 antibodies recognize the complete set of AChE variants. According to previous findings [13], the labelled bands were assigned to the synaptic AChE (variant T; 68 kDa) and to the read-through AChE-R (50 kDa). Note the weaker labelling of the catalytically active AChE subunit (68 kDa) in SCC (S) and the stronger labelling of the 50 kDa protein band in tumoral ASF. The right-hand panel shows the correlation between AChE activity loaded and the intensity of labelling.

The results of the present study show that human lung epithelium expresses the components for establishing a physiologically active cholinergic system, possibly needed for correct pulmonary function and involved in cancer growth. The present study shows that: (i) ASF contains enough AChE activity to control the effective levels of the neurotransmitter and/or its degradation product choline; (ii) AChE activity varies according to the histological type of the lung tumour, being highest in ASF from lung ACs and lowest in SCLCs; (iii) the pattern of molecular forms of AChE do not change with cancer; (iv) inactive AChE molecules are present in bronchial aspirates, as in lung tissues, increasing their content in cancerous samples and having histological-type differences; and (v) both SCLC and NSCLC cells express the genes needed for a cholinergic autocrine or paracrine loop.

It has been clearly demonstrated that nicotine and other tobacco components increase the proliferation of non-cancerous and cancerous human lung cell lines [6,18,33,34], at the same time as ACh or carbachol also modulated the proliferation of lung cells [6,35]. By using agonists or antagonists of nAChR and mAChR, the function. Furthermore, it appears likely that nicotine plays its pathological role through direct interaction with cholinergic receptors [18,20,37]. Previous studies elucidating the events implicated in these receptor-dependent responses [6,17,36–39] are important not only for tobacco-consumption-associated cancer, but also for lung tumours, such as AC, that have a weaker correlation with smoking than SCC and SCLC.

According to RT–PCR, our results are consistent with others showing that lung cells express the mRNAs needed for establishing functional cholinergic systems in both SCLC and NSCLC cells (reviewed [1,2]). Nevertheless, as far as we know, there is no association between particular cholinergic gene expression patterns and the pathogenesis of lung cancer. A very interesting study by Minna and co-workers [40] shows that tobacco consumption could change the composition of mAChRs on bronchial epithelial cells. It remains to be seen whether
these changes are important for the pathogenesis of lung cancers, but they clearly indicate the existence of particular nAChR subunit gene expression patterns which change upon several physiological conditions. As ACh levels could also change with other pathologies, for example in response to inflammatory processes [1,41], the same pathways that are involved in nicotine-associated lung cancer progression could also play a role in the biology of lung cancer not linked to tobacco consumption.

Our present results show that ASF contains an important amount of AChE activity, which varies depending on the histology of the lung carcinoma (Table 2). AChE activity in ASF was higher than the control in AC, and lower in SCLC and SCC. We have shown previously that surgical fragments from SCC have considerably less AChE activity than non-cancerous and AC samples [13]. It is interesting that ASF from smoking-associated lung cancers, such as SCC and SCLC, contains less AChE activity than that from controls or AC. The actual role of AChE activity in cancer is controversial. Cancer promotes changes affecting both AChE gene structure and expression [42,43] and the properties of the protein [13,44–47]. More recently, it has been proposed that AChE plays a role in apoptosis [26–28], whereas immunohistochemical staining is inversely associated with survival in ovary cancer [48].

AChE biology is a very complex process and the precise physiological role of this enzyme in non-neuronal contexts is poorly understood. AChE can be found as soluble or membrane-bound subunits, but little is known about its regulation and physiological significance. Whether secretion of AChE molecules into the epithelial lining fluid of the bronchi is a controlled process needed for normal pulmonary function needs to be investigated further. Thus it cannot be assumed, but also it cannot be excluded, that a correlation exists between AChE activity in the fluid of aspirates and in malignant epithelium cells. A correlation between AChE activity in both ASF and surgical fragments in SCCs with a large decrease in the AChE activity was found, whereas no correlation between AChE activity in ASF and surgical fragments derived from ACs was observed. We discussed in our previous study [13] that the heterogeneous cellular origin of lung cancers included in the AC group may mask possible differences, and the same can happen with the results of AChE activity in ASF. It is tempting to speculate that in AC the secretion of active AChE is up-regulated and that this phenomenon affects the biology of the tumour. On the other hand, the differences between AChE activity in ASF from AC and SCLC could be of diagnostic interest.

With regard to the molecular composition of AChE, sedimentation analysis revealed that ASF contains abundant amphiphilic dimers (G2A) and fewer monomers (G1A) (Figure 1), the same pattern as in the cellular fraction of bronchial aspirates. The similarity between the AChE
pattern in ASF and the expression by tumours of epithelial origin, such as lung [13], breast and metastatic lymph nodes [44,45] and colon [49], reinforces the idea that human epithelia only express the light forms of AChE as enzymatic active forms. As human serum contains a very small amount of AChE activity and high BChE activity and the opposite is found in ASF, it is also possible that ASF AChE comes from the bronchial epithelium. The pattern of molecular forms of AChE from cultured cancer lung cells also shows that only SCLC cells express AChE tetramers, and this appears to be related to adhesion-independent growth, but not to the histological type.

Immunoblotting results indicate that the same labelled protein bands are found in ASF (Figure 2) and the cellular fraction of bronchial aspirates (results not shown). It is remarkable that ASF contains both inactive AChE molecules, as occurs in lung tissue (50 kDa protein band), and active AChE subunits (approx. 70 kDa). In agreement with our previous study [13], SCC had the lowest content of active subunits (Figure 2, lane S). We have previously assigned the 50 kDa protein band to AChE-R, the stress-induced AChE variant, which is synthesized more abundantly in lung cancer tissues and especially in SCC. Interestingly, Soreq and co-workers [50] found that basal levels of CREB (cAMP-response-element-binding protein) were insufficient to block the proliferative effect of AChE-R in AChE-R-transfected glial cells and they hypothesized that this could increase the risk of tumour growth in individuals exposed to anticholinergic agents. The potential diagnostic use derived from the present study and the physiological significance of cholinesterases in lung cancer warrants further investigation.

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