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Cholinesterase activity of human lung tumours varies according to their histological classification

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The probable involvement of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in cancer and the relevance of cholinergic responses for lung cancer growth prompted us to study whether cholinesterase activity of human lung is altered by malignancy. Surgical pieces of non-small lung carcinomas (NSLC) and their adjacent non-cancerous tissues (ANCT) were analysed for AChE and BChE activities. AChE activity in adenocarcinoma (AC) was 7.80 ± 5.59 nmol of substrate hydrolysed per min and per mg of protein (mU/mg), the same as in their ANCT (8.83 ± 4.72 mU/mg; $P = 0.823$); in large cell carcinoma (LCC), 7.52 ± 3.32 mU/mg, ∼50% less than in their ANCT (15.39 ± 5.66 mU/mg; $P = 0.043$); and in squamous cell carcinoma (SCC), 1.39 ± 0.58 mU/mg, 80% less than in ANCT (6.08 ± 2.88 mU/mg; $P = 0.003$). BChE activity was 5.85 ± 3.20 mU/mg in AC and 9.56 ± 3.38 mU/mg in ANCT ($P = 0.022$); 2.94 ± 2.01 mU/mg in LCC and 6.50 ± 6.63 mU/mg in ANCT ($P = 0.068$); and 4.49 ± 2.30 mU/mg in SCC and ANCT 6.56 ± 4.09 mU/mg ($P = 0.026$). Abundant AChE dimers and fewer monomers were identified in lung and, although their distribution was unaffected by cancer, the binding with concanavalin A revealed changes in AChE glycosylation between SCC and their ANCT. The fall in BChE activity affected all molecules, with a strong decrease of the amphiphilic tetramers. Western blotting revealed protein bands with the expected mass of the principal AChE subunits, and the deeper intensity of the protein signal in SCC than in healthy lung, in lanes loaded with the same units of AChE activity, supported an augment in the amount of AChE protein/unit of AChE activity in SCC. The increased availability of acetylcholine in neoplastic lung, resulting from the fall of cholinesterase activity, may enhance cholinergic signalling and contribute to tumour progression.

Abbreviations: AC, adenocarcinoma; ACh, acetylcholine; AChE, acetylcholinesterase; ANCT, adjacent non-cancerous tissue; BChE, butyrylcholinesterase; ChE, cholinesterase; ChAT, choline acetyltransferase; GPI, glycosylphosphatidylinositol; LCC, large cell carcinomas; NSCLC, non-small cell lung carcinomas; SCLC, small cell lung carcinomas; SCC, squamous cell carcinomas.

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Introduction

Lung cancer is a leading cause of death due to cancer worldwide and it is expected to cause 2 000 000 deaths per annum by the years 2020–2030 (1). On the basis of morphological criteria and clinical outcome, the Word Health Organization (WHO) classifies lung cancers into small cell lung carcinomas (SCLC) and non-small cell lung carcinomas (NSCLC) (2). SCLC account for 20–25% of lung cancers; they are tightly linked to smoking (3) and develop and metastasize rapidly. NSCLC tend to grow and spread more gradually than SCLC and, according to histological features, NSCLC are classified into adenocarcinomas (AC), squamous cell carcinomas (SCC) and large cell carcinomas (LCC).

While the survival rate for non-lung cancers has notably improved in the last decade, the lung cancer 5 years survival rate is still very low (13–15%) despite innovations in diagnostic testing, surgical techniques and chemotherapeutic treatments. The poor outcomes of lung cancer, when compared with other tumours, can be related to fundamental differences in bronchopulmonary tumour biology. A better understanding of the molecular mechanism leading to lung cancers would open the way to new therapies for improving patients' survival and quality of life.

SCLC and many NSCLC show neuroendocrine features, including production of neuropeptides, which together with the expression of cell surface receptors, lead to growth loops (4). Lung development (5) and cancer growth (6,7) require cholinergic signalling and the mitogenic effects of nicotine on lung cells have been attributed to cholinergic responses. By acting through autocrine or paracrine loops, acetylcholine (ACh) possibly alters the growth of lung cells.

In the last few years, non-neuronal cholinergic systems have been identified in human tissues consisting of epithelial, endothelial and mesothelial cells, as well as in muscle fibres and glial, parenchymal and immune cells (8). Experimental evidence supports the involvement of non-neuronal cholinergic systems and non-neuronal ACh in myophaties (9), skin pathologies (10,11) and inflammatory and autoimmune diseases (8). Such a variety of disorders suggests multiple functions for non-neuronal cholinergic systems, each function possibly depending on the particular cell phenotype.

Cholinergic systems contain the set of proteins required for ACh synthesis (principally choline acetyltransferase, ChAT), signal transduction (nicotinic and muscarinic receptors, nAChR and mAChR), acetylcholine hydrolysis (cholinesterases, ChEs) and choline uptake (high-affinity choline transporters, HACT). Vertebrates contain two ChEs, acetylcholinesterase (AChE; acetylcholine acetylhydrolase, EC: 3.1.1.7) and butyrylcholinesterase (BChE; acylcholine

acylhydrolase, EC: 3.1.1.8). AChE prefers ACh as the substrate and its function is critical for terminating cholinergic responses. BChE preferentially hydrolyses butyryl- and succinyl-choline (12), and it can replace AChE for ACh clearance when AChE is inhibited or absent (13). The existence of ChEs in non-excitable tissues, blood cells and body fluids supports their involvement in cellular functions other than catalysis (12,14–17).

The human *AChE* gene maps at chromosome 7 (7q22) (18) and the *BChE* gene at chromosome 3 (3q26) (19). Their protein products occur in tissues as polymers of catalytic subunits. The range of AChE molecules arises from mRNA splicing, post-translational changes, assembly of catalytic subunits and attachment of structural (non-catalytic) subunits (20,21). Three alternative exons define the C-terminal domain of the AChE subunits. The AChE-T (from tailed) transcript arises from splicing of exon 4 (E4) to E6; it encodes the AChE-T protein subunit, also called synaptic or S-subunit. The AChE-H (from hydrophobic) mRNA arises by joining E4 and E5; it yields the glycosylphosphatidylinositol (GPI)-linked AChE subunit. The continuous transcription through intron I4 yields the AChE-R transcript (from read-through), which produces the AChE-R subunit. New N-terminus extended AChE subunits (N-AChEs) have recently been identified, whose expression is developmentally regulated in neurons and blood mononuclear cells (22). A single *BChE*-mRNA has been observed so far.

Experimental evidence supports the involvement of ChEs in cancer. Thus, aberrations of the *AChE* gene are common in leukaemia (23) and ovarian carcinomas (24), and human tumour cell lines express anomalously spliced AChE-mRNAs (25). In addition, changes in the structure and/or expression of the *AChE* gene as well as the presence of ChE molecules with abnormal properties have been reported in brain and non-brain tumours (26–28). In the context of ChEs in cancer, it is worth mentioning the opposite variation of AChE and BChE activities in human breast carcinomas (29), the great drop of AChE activity in lymph nodes affected by breast cancer metastasis (30), the association between the change in the composition of AChE variants and the aggressiveness of human astrocytoma (31) and the active participation of AChE in apoptosis (32).

Insights into the possible involvement of ChEs in lung carcinogenesis were gained by studying for the first time the effects of cancer on bronchopulmonary ChE activity, the molecular distribution and the level of ChE protein. The results show that lung contains a moderate level of ChE activity, which is greatly reduced in SCC. The variation of ChE activity in the three classes of NSCLC can be related to differences in the biology of the lung cells from which each kind of cancer arises.

Materials and methods

Chemicals

Acetylthiocholine iodide, butyrylthiocholine iodide, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), 1,5-bis (4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284c51), tetraisopropyl-pyrophosphoramide (*iso*-OMPA), proteinase inhibitors, polyoxyethylene$_{10}$-oleyl ether (Brij 96), enzyme markers for sedimentation analysis (bovine liver catalase, 11.4S, $S_{20,w}$, Svedberg Units, and bovine intestine alkaline phosphatase, 6.1S), lectin-free Sepharose 4B and agarose-bound lectins (concanavalin A, Con A; *Lens culinaris* agglutinin, LCA; and wheat germ agglutinin, WGA) were all obtained from Sigma (Madrid, Spain). Goat antisera against the N-terminal peptide (N19) and against the C-terminal peptide (C16) of human AChE were purchased from Santa Cruz Biotech (Santa Cruz, CA) and antisera against human BChE were purchased from Accurate Chemicals (Westbury, NY). According to the manufacturers' information, the C16 antiserum was raised against a C-peptide of human AChE differing from the homologous mouse sequence by a single amino acid. A comparison of the human and mouse C-terminal sequences for the AChE-T, -H and -R subunits let us conclude that C16 antiserum is produced against the C-terminus of the human AChE-T. Anti-goat IgG monoclonal antibodies conjugated with alkaline phosphatase were provided by Sigma. Molecular weight protein standards (SDS-6H and BenchMark), for measuring the mass of AChE subunits, were acquired from Sigma and Invitrogen, respectively. For RT–PCR assays Total RNA purification system (Invitrogen, Carlsbad, CA, USA) and GeneAmp RNA PCR Kit (Applied Biosystems, Branchburg, NJ, USA) were used. Other chemicals were of analytical grade.

Patients and tumours

A total of 31 specimens of primary NSCLC and adjacent non-cancerous tissue (ANCT) were taken from patients undergoing surgery at the University Hospital *Virgen de la Arrixaca* in Murcia (Spain) between 2001 and 2004. Of the 31 patients, 28 were male and 3 female, with ages ranging from 45 to 79 years and the mean age being 63.9 years. Patients were not subjected to any chemotherapeutical treatment before surgery. The malignant samples weighed 28–431 mg and the ANCT pieces 34–222 mg. The specimens were frozen and stored at −80°C until required. A histological evaluation of the tumours, applying the WHO criteria, revealed that 14 corresponded to AC, 6 to LCC and 11 to SCC. Staging was assessed according to the TNM classification system (Tumour Node Metastasis Staging System). All patients were appropriately informed and gave their consent.

Extraction and assay of ChE activity and ACh content

AChEs and BChEs in unaffected and neoplastic pieces were extracted as described previously (28). Lung samples were homogenized (5% w/v) in Tris–saline buffer (TSB; 1 M NaCl, 50 mM $MgCl_2$, 3 mM EDTA and 10 mM Tris, pH 7.5) containing a fresh mixture of proteinase inhibitors (0.1 mg/ml soybean trypsin inhibitor, 1 mg/ml bacitracine, 0.0022 TIU/ml aprotinin, 10 μg/ml pepstatin A and 20 μg/ml leupeptin). After centrifugation at 30 000 r.p.m. in a TLA 100.4 Beckman rotor, 35 min at 4°C, the S1 supernatant with soluble and loosely bound ChEs was saved. The pellet was extracted again with TSB containing the cocktail of antiproteinases and Brij 96 (1% w/v) and, after centrifugation as above, the S2 supernatant with membrane-bound ChEs was recovered.

AChE and BChE activities were measured by the Ellman method (33) as described previously (29). AChE was assayed with 1 mM acetylthiocholine and 50 μM *iso*-OMPA and BChE with 1 mM bytyrylthiocholine and 10 μM BW284c51. One unit (U) of ChE activity represents the hydrolysis of 1 μmol of substrate per minute at 37°C. The contribution of unspecific esterases to the hydrolysis of substrate was estimated by including both BW 284c51, an inhibitor of AChE, and *iso*-OMPA, an inhibitor of BChE, in the assay mixture. The hydrolysis of substrate due to unspecific esterases in lung extracts was negligible (0–11% of the hydrolysis due to ChE). AChE and BChE activities in fractions collected from sucrose gradients were determined by a microtiter assay in 96-well plates (34), in which case the activity is given in arbitrary units, one unit referring to an increase of 0.001 absorbance units/μl of sample/min. The amount of protein was estimated by the Bradford method (35) with bovine serum albumin (BSA) as the standard.

The amount of ACh was measured using the Acetylcholine assay kit (Amplex Red; Molecular Probes). This kit measures the amount of hydrogen peroxide produced through the oxidation of choline. Separation of endogenous AChE from ACh was achieved by filtration with Amicon® Ultra centrifugal filter devices (Amicon ultra-4 10 000 MWCO, Millipore). For the measurement of ACh and choline, 0.1 ml each of filtered homogenates were spotted in duplicate onto 96-well microplates. An ACh standard curve (0–10 μM) was used in each experiment. In each well, 0.1 ml of assay buffer (50 mM Tris–HCl, pH 7.5) containing 0.2 M Amplex Red reagent, 2 U/ml horseradish peroxidase, 0.2 U/ml choline oxidase and 10 U/ml AChE was added. To measure the amount of choline, AChE was omitted from the assay buffer. After incubation (15 min.), the fluorescence was determined in a fluorescence microplate reader using 530 nm excitation wavelength and 590 nm emission wavelength. ACh content was calculated from the difference between the ACh plus choline and choline concentration.

Sedimentation analysis

Lung AChE and BChE molecules were resolved and identified by their sedimentation coefficients. Samples and sedimentation markers (bovine liver catalase and intestine alkaline phosphatase) were layered on the top of centrifuge tubes containing 5–20% sucrose gradients prepared with 0.5% Brij 96 and

TSB. The gradient tubes were centrifuged at 35 000 r.p.m. in a SW41Ti Beckman rotor (Fullerton, CA, USA), 18 h at 4°C, and after centrifugation the fractions (275 μl) were collected from the tube bottom and assayed for ChE activity and enzyme markers.

Binding of AChE with immobilized lectins

Insights into the possible effect of cancer on the sugar composition of the oligoglycans linked to lung ChEs were gained by testing enzyme interaction with several plant lectins. For this, equal volumes of S1 plus S2 were mixed and incubated overnight at 4°C with lectin-free Sepharose 4B (control) and with agarose-bound lectins (Con A, LCA and WGA). The agarose beads were separated by centrifugation and the unbound AChE and BChE activities were measured in the supernatants. ChE activity in control assays (those made with lectin-free Sepharose 4B) was taken as the 100% value and the percentage of lectin binding was calculated from the difference between the activity in control and lectin-incubated supernatants.

Western blotting

AChE and BChE subunits in healthy and malign lung were resolved by reductive SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5% acrylamide gels (36). Proteins were electro-transferred to nitrocellulose sheets, blocked with 3% BSA and incubated with anti-AChE (N19 and C16) or anti-BChE antisera. Since N19 antibodies are produced against the N-terminal peptide of human AChE, it should react with the full set of AChE variants. In contrast, the C16 antiserum, raised against a C-terminal sequence of the AChE-T subunit, should only recognize molecules made of T subunits. Labelled proteins were revealed with anti-goat IgG alkaline phosphatase-conjugated antibodies and an appropriate substrate (BCIP/NBT). The size of ChE subunits was estimated by using suitable protein standards, and the intensity of the protein bands was quantified with the GelPro Analyzer Software (version 3.1; Media Cybernetic).

Reverse transcriptase–polymerase chain reaction

RT–PCR was used to investigate the expression of AChE and BChE RNAs in lung tissues. Total mRNA was extracted from ANCT and cancerous lung pieces using the Total RNA purification kit (Invitrogen), and 1 μg of RNA was reverse transcribed into cDNA by random priming (GeneAmp RNA PCR Kit, Applied Biosystems). The primers used to detect the mRNA of the synaptic AChE (AChE-T) were as follows: forward, 5′-AACTTTGC-CCGCACAGGGGA and reverse, 5′-GCCTCGTCGAGCGTGTCGGT. For the AChE-mRNA encoding the glycophospholipid-anchored AChE-H the primer pair was as follows: forward, 5′-AACTTTGCCCGCACAGGGGA and reverse, 5′-GGGAGCCTCCGAGGCGGT. The read-through AChE-mRNA (AChE-R) was amplified by using forward 5′-CCCCTGGAC-CCCTCTCGAAAC and reverse 5′-ACCTGGCGGGCTCCCACTC primers. A novel 5′ variant of AChE-mRNA (E1d; ref. 22) was detected by PCR with forward (5′-CCTGGTGACGAAAGTCCGA) and reverse (5′-TCCTCCAC-CCAGGAGCCAGAG) primers. To detect the BChE-mRNA, we employed forward (5′-TGTCTTTGGTTTACCTCTGGAA) and reverse (5′-CACT-CCCATTCTGCTTCATC) primer pair. Finally, forward (5′-AGAAAAT-CTGGCACCACACC) and reverse (5′-GGGGTGTTGAAGGTCTCAAA) primers were used to generate a fragment of β-actin-mRNA as internal control. RT–PCR products were electrophoresed on 1.5% agarose gels.

Statistical analysis

The results are given as mean ± SD. The statistical significance of the difference in ChE activity between ANCT and each histological type of lung cancer (AC, LCC and SCC) was evaluated by the non-parametric Wilcoxon matched-pairs signed-ranks test at $P < 0.05$. The same test was used to explore whether the difference in ChE activity between adjacent healthy tissues and the various kinds of cancers was correlated with clinicopathological parameters. The statistical significance of the difference in AChE and BChE activities between the three types of lung tumours was evaluated by the non-parametric Mann–Whitney *U*-test ($P < 0.05$). Statistical analysis was performed using the SPSS software program (version 10 for Windows; SPSS, Chicago, IL).

Results

AChE and BChE activities and ACh content in unaffected and cancerous lung

The measurement of ChEs in healthy and cancerous lung samples (Table I) showed a higher AChE activity in ANCT to LCC than to AC or SCC. This variation possibly arises from differences in the cellular composition of lung pieces. As for cancerous samples concerns (Table I), AChE activity was unaffected in AC, it dropped by ∼50% in LCC and by ∼80% in SCC, which indicated that the change of AChE activity in lung cancers depends on the particular biology of the epithelial cell type which originates the tumour. The great reduction of AChE activity in SCC may play a role in the biology of squamous cell in contrast to AC. BChE activity was notably greater in ANCT than in AC or SCC (Table I). The difference between BChE activity in LCC and ANCT was almost statistically significant ($P = 0.068$), but only four samples were available for BChE measurement. The decreased levels of both AChE and BChE activities in LCC and SCC (Table I) besides the strong reduction of AChE and the maintenance of BChE activity in metastasized lymph nodes (30) discard that the fall in lung AChE is compensated by BChE activity and suggest that the two enzymes are independently regulated by cancer.

Table I. AChE and BChE activity in ANCT and lung carcinomas

Wilcoxon test	AChE activity (*n*) (mU/mg)	BChE activity (*n*) (mU/mg)	*P*-value	
			AChE	BChE
AC	7.80 ± 5.59 (14)	5.85 ± 3.20 (10)	0.875	0.022
ANCT	8.83 ± 4.72 (14)	9.56 ± 3.38 (10)		
LCC	7.52 ± 3.32 (6)	2.94 ± 2.01 (4)	0.043	0.068
ANCT	15.39 ± 5.66 (6)	6.50 ± 6.63 (4)		
SCC	1.39 ± 0.58 (11)	4.49 ± 2.30 (11)	0.003	0.026
ANCT	6.08 ± 2.88 (11)	6.56 ± 4.09 (11)		
Mann–Whitney *U*-Test	*P*-value	*P*-value		
AC vs LCC	0.823	0.203		
AC vs SCC	0.000	0.260		
LCC vs SCC	0.002	0.240		

AChE and BChE activities are given as mean ± SD (number of samples). One unit of ChE activity represents the hydrolysis of 1 μmol of the substrate per minute at 37°C. The statistical difference in AChE or BChE activities between ANCT and cancerous samples (AC, LCC and SCC) was assessed by the non-parametric Wilcoxon test for paired samples ($P < 0.05$). The statistical significance of the difference in ChE activity between the three kinds of tumours was evaluated by the non-parametric Mann–Whitney *U*-test ($P < 0.05$)

The possible use of ChE activities for distinguishing the histological type of lung tumours was explored by comparing the variation of AChE activity between the three kinds of lung cancers (Table I). Application of the non-parametric Mann–Whitney *U*-test revealed that the change of AChE activity was statistically significant between AC and SCC and between LCC and SCC, but not between AC and LCC. No correlation was observed between the change of AChE activity in lung cancers and clinicopathological factors, including patient's gender and age, tumour size, histological grade and lymph node status.

Most of the ChE activity in unaffected and cancerous lung was brought into solution by the two-step extraction method. About half of AChE activity in LCC (45.6 ± 4.2%) and SCC (41.2 ± 19.6%), and somewhat less activity in AC (28.1 ± 10.9%) and ANCT (33.6 ± 15.9%), was released with saline buffer alone (S1), and the rest with Brij 96 (S2). In contrast, the majority (81.9 ± 13.7%) of BChE activity was extracted with saline buffer. Since the extent of AChE release in S1 denotes the binding strength of the enzyme to cell membranes, the

Table II. Acetycholine content in adjacent non-cancerous tissue (ANCT) and lung carcinomas

Samples	ACh (*n*) (μM)	*P*-value
AC	1.44 ± 0.46 (11)	0.015
ANCT	0,98 ± 0.58 (7)	
LCC	1.09 ± 0.59 (4)	0.029
ANCT	0.42 ± 0.13 (4)	
SCC	1.57 ± 0.51 (10)	0.014
ANCT	1.01 ± 0.44 (9)	

ACh content is given as mean ± SD (number of samples). The statistical difference in ACh content between ANCT and cancerous samples (AC, LCC and SCC) was assessed by the non-parametric Mann–Whitney *U*-test ($P < 0.05$).

variable percentages of AChE extraction, according to the histological type of the tumour, may reflect structural changes in the surface membrane of malignant cells or an increase in the cellular damage.

The measurement of ACh in non-cancerous and the three histological kinds of NSCLC (Table II) showed that lung cancers had a higher content of ACh than their ANCTs.

Identification of AChE molecules in human lung

The molecular distribution of AChE and BChE in lung and its possible impairment by cancer were studied by sedimentation analysis. For this, AChE and BChE forms in the S1 and S2 extracts of ANCT and cancerous lung were resolved by centrifugation analysis and identified by their sedimentation coefficients. Sedimentation profiles showed principal ∼4.0S and minor ∼2.5S AChE molecules in S1 and S2 extracts of ANCT (Figure 1). According to previous data (28), the molecules were assigned to amphiphilic AChE dimers (G_2^A; 4S) and monomers (G_1^A; 2.5S). Occasionally, 6.0S and 9.5S variants, possibly hydrophilic dimers (G_2^H) and amphiphilic tetramers (G_4^A), were also identified, but their contribution, when any, was scarce. ANCT and the three kinds of lung carcinomas gave similar patterns of AChE forms, indicating that whatever the histological abnormalities of lung carcinomas are they do not affect the biosynthesis of lung AChE molecules.

Abundant hydrophilic G_4 BChE and fewer G_2 and G_1 molecules were identified in S1 of ANCT and the three types of lung carcinomas. In S2 extracts of ANCT, G_4^H, G_4^A, G_2^A and G_1^A BChE forms were observed, and this differs from the molecular pattern of cancerous samples, where an almost complete loss of the G_4^A components was found (Figure 1).

Effects of cancer on the oligoglycans linked to lung AChE

Lectin interaction assays allowed us to investigate the possible effects of cancer on the oligosaccharides of lung AChE and BChE. A great fraction (85-100%) of AChE activity in ANCT, AC, LCC and SCC was bound by LCA and WGA, and no statistically significant differences in the percentage of binding between the above lectins and AChE in the various types of lung samples were observed (Figure 2). However, the assays with Con A revealed important differences. Thus, while Con A retained 50–60% of the AChE activity in ANCT, AC and LCC, the lectin binding extent increased to nearly 95% (93.2 ± 6.3) for the SCC enzyme. Most of the lung BChE was bound to the lectins and no differences in the binding extent were observed between healthy and cancerous pieces.

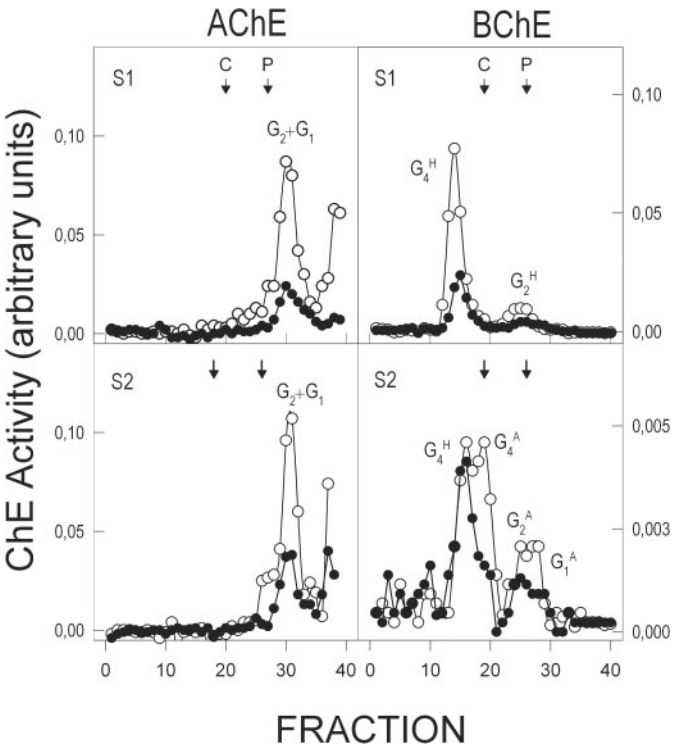

Fig. 1. Representative sedimentation profiles of AChE and BChE in human lung. Tissues were extracted with Tris-saline buffer (TSB) and proteinase inhibitors, and after centrifugation the soluble and weakly bound AChE was saved in the S1 supernatant. The pellet was extracted again with TSB, proteinase inhibitors and 1% Brij 96, and after centrifugation tightly bound AChE was obtained in S2. AChE forms in S1 and S2 were identified by sedimentation analysis in sucrose gradients (5-20%) containing 0.5% Brij 96. The internal sedimentation markers were catalase (C; 11.4S) and alkaline phosphatase (P; 6.1S), and the sedimentation coefficients of AChE and BChE components were calculated by taking these as the reference. The absence of significant differences between the profiles of AChE forms in extracts of adjacent non-cancerous (open circle) and cancerous lung (closed circle) demonstrates that the synthesis of lung AChE molecules is not affected by cancer. In contrast, the fraction of the G_4^A BChE species is greatly reduced in cancerous lung.

Immunoblot analysis

Possible size differences between ChE subunits of ANCT and malignant lung pieces as well as the presence in them of catalytically inactive AChE and/or BChE subunits were explored by western blotting. For this, a mixture of S1 + S2 extracts from control and neoplastic tissues, containing the same units of AChE activity or the same amount of protein, were subjected to reductive SDS-PAGE and then incubated with the N19 or C16 antisera (Figure 3). Protein bands of 72, 68, 55 and 50 kDa were observed in ANCT and lung tumour samples blotted with N19 (Figure 3A and B), their sizes coinciding with those reported for human AChE subunits (37). Only the 68 and 55 kDa proteins were marked with C16 antibodies (Figure 3C) and, since they specifically recognize the C-terminus of the AChE-T subunit, the 68 kDa protein was tentatively assigned to the T subunit and the 55 kDa to a lytic derivative. The 72 kDa protein may correspond to the AChE-H subunit, or alternatively to a glycovariant of the AChE-T subunit. Finally, the 50 kDa protein probably represents a lytic fragment of the AChE-R subunit (the stress-related AChE variant; see ref. 37) and, if so, its deeper labelling in the SCC than in the control sample, in spite of the equivalent

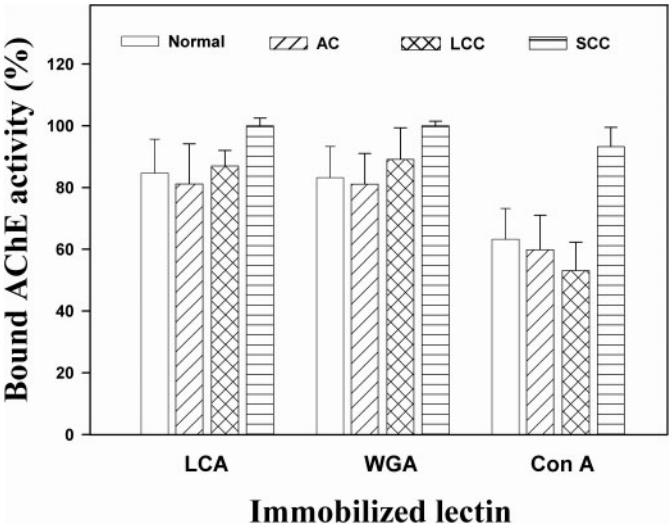

Fig. 2. Lectin binding with AChE activity of non-cancerous lung (normal), AC, LCC and SCC. A mixture of the S1 and S2 supernatants was incubated with lectin-free Sepharose 4B (control) or with immobilized lectins. After incubation overnight at 4°C, the lectin–glycoprotein complexes were removed by centrifugation, and free AChE activity was assayed in the supernatant. The bars show the percentage of binding of AChE with the lectins. AChE activity in control experiments was taken as the 100% value. Results are means of at least five experiments made with adjacent non-tumorous and cancerous samples (*, $P = 0.009$).

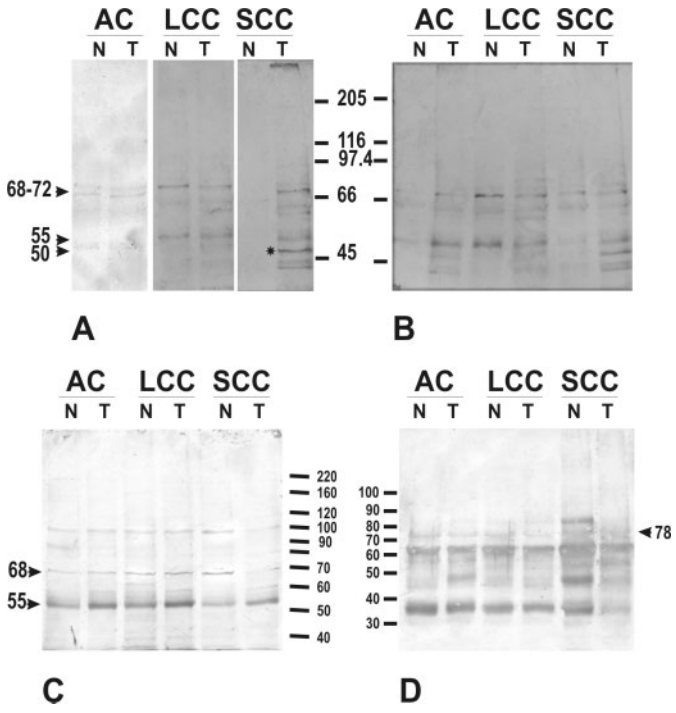

Fig. 3. Subunit size and relative content of AChE protein in normal and tumoral lung. Proteins in ANCT (normal; N) and tumoral (T) lung were resolved by reductive SDS-PAGE in 7.5% acrylamide gels. After transferring to nitrocellulose sheets, proteins were blotted with N19 antibodies, against the AChE protein core, or with C16 antibodies, against the C-terminal peptide of AChE-T. Sheets (**A**) and (**B**) were incubated with N19, sheet (**C**) with C16 and sheet (**D**) with anti-BChE antibodies. In sheet (A), the N and T lanes were loaded by pairs with 0.2 (AC), 0.3 (LCC) and 0.07 (SCC) mU of AChE activity; an equal amount of protein (30 μg) was loaded in the corresponding lanes of sheets (B) and (C). According to previous data (36), the labelled bands were assigned to the synaptic AChE-T subunit (68 and 55 kDa), the GPI-linked AChE-H subunit (72 kDa) and the read-through AChE-R subunit (50 kDa). Note the deep labelling of the 50 kDa protein in the SCC sample (represented by an asterisk). Since N and T lanes of sheet (A) contain equivalent units of AChE activity, the heavier labelling in the T-SCC lane means that the amount of AChE protein/unit of AChE activity increases in SCC, an issue confirmed by the strong signal in the T-SCC lane of sheet (B). The BChE protein (78 kDa) was similarly stained in ANCT and cancerous samples.

units of activity loaded in wells, may reflect an increased content of the stress-related AChE subunit in lung SCC.

A comparison of the labelling intensity of AChE protein bands in paired samples of normal and cancerous tissues (Figure 3), in lanes loaded with equivalent units of AChE activity, revealed a stronger labelling in SCC samples, which pointed to the existence of inactive AChE in lung and to a higher amount of AChE protein per unit of AChE activity in SCC than in ANCT, LCC or AC samples.

An immunoblotting with labelled BChE is shown in Figure 3D. The 78 kDa protein band was assigned to BChE subunits and, in contrast with AChE, no significant differences in the labelling intensity between ANCT and cancerous tissues were observed.

Expression of AChE- and BChE-mRNAs

Both non-cancerous lung and the three types of bronchopulmonary tumours produced the 3′alternative AChE-mRNA trancripts (T, H and R) and the BChE-mRNA (Figure 4). Furthermore, we tested the expression of AChE-mRNAs containing an alternative first exon as a result of a different promoter usage (named as E1d-AChE-mRNA by Soreq's group; ref. 22). E1d-AChE-mRNA was identified in four out of five ANCT samples, and its absence from the set of cancerous pieces studied (three samples of AC and two of SCC) can be useful for diagnostic purposes.

Discussion

We report here, for the first time, valuable information concerning AChE and BChE activities in human lung, the distribution of enzyme molecules, the interaction with lectins and the size of enzymatic subunits. In addition, details regarding the change of ChE activities in various kinds of lung tumours and the effects of cancer both on the glycosylation of AChE and on the amount of immunolabelled AChE protein are also given.

Our results show that human lung contains an important level of AChE activity (between 6.1 and 15.4 mU/mg protein), which is lower than lymph nodes (21 mU/mg; ref. 30), roughly the same as gliomas (∼13 mU/mg; ref. 38) and neurinomas (∼9 mU/mg; ref. 39), and greater than breast (∼1.6 mU/mg; ref. 29). The finding of AChE activity in human lung agrees with previous data showing its presence in pig airways and bronchial epithelial cells, where a role for AChE in the control of airway muscle contraction has been proposed (40). Moreover, the existence of AChE activity in lung joined to the observation in lung and tumour cell lines of the complete protein set which forms the non-neuronal cholinergic system (6,41) demonstrate that pulmonary epithelium can respond to cholinergic signalling. Accordingly, it has been postulated that the non-cholinergic system of airways participates in the regulation of lung cell phenotypes (41). As regards BChE, human lung epithelium contains more BChE activity than brain (1.5 mU/mg; ref. 34), almost the same as neurinomas

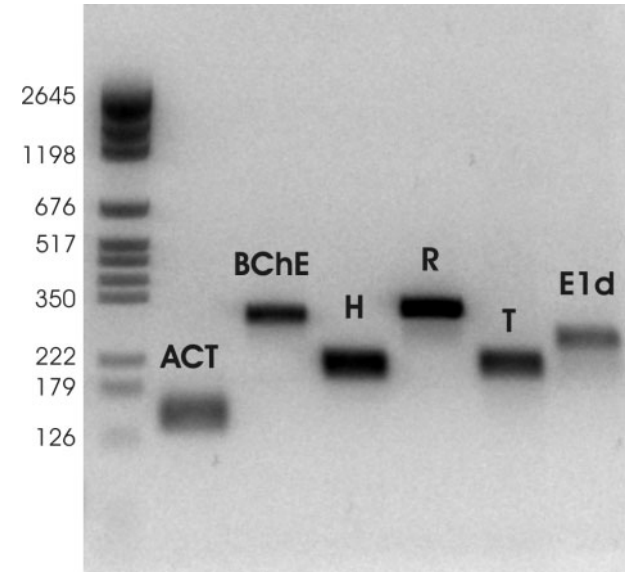

Fig. 4. Identification of AChE- and BChE-mRNAs in lung. Total RNA was isolated, 1–2 ug were reverse transcribed, and the resulting cDNA was amplified with primer pairs for BChE, AChE-H (H), AChE-R (R), AChE-T (T) and E1d-AChE-mRNAs. β-actin-mRNA (ACT) was used as the control.

(4.6 mU/mg; ref. 39) and less than gliomas (15.2 mU/mg; ref. 38).

Owing to cancer, AChE activity dropped by ~80% in LCC, by 50% in SCC and by nothing in AC (Table I), demonstrating that AChE activity changes according to the histological features of lung tumours. The heterogeneous cellular origin of lung cancers included in the AC group (acinar, papillary, solid carcinoma with mucus production) may mask any possible difference between AChE activity in healthy lung and in a particular AC subtype. BChE activity fell by 40–50% in AC, SCC and LCC extracts (Table I).

The drop of AChE activity in LCC, SCC and metastasized lymph nodes (30) could lead to the belief that, regardless of the cellular origin, the *AChE* gene is always downregulated in neoplastic tissues. However, the absence of any noticeable change in AChE activity from AC and its increase in malignant breast (29) rule out the above contention and suggest that the effect of cancer on AChE activity depends on the genetic and biological abnormalities developed in the particular kind of cell from which the tumour arises.

The involvement of AChE in the hydrolysis of lipid peroxides (42) raises the possibility that the fall in the enzyme activity enhances oxidative stress and cellular damage of bronchopulmonary epithelial cells. In addition, the decrease of AChE activity in LCC and, especially, in SCC may contribute to tumour development by increasing cell growth or proliferation. In this regard, the importance of cholinergic signalling for lung physiology, the mitogenic action of nicotine on SCLC and the actions of cholinergic antagonists on the growth of cultured lung cells should be reminded of (6,43). The activation of the phosphatidylinositol-3 kinase/Akt signalling pathway in lung epithelial cells by both nicotine and the nicotine-derived carcinogenic nitrosamine, NNK, (7,44,45) demonstrates the relationship between cholinergic stimulation and lung cancer. In addition, the results showing the linkage between the binding of ACh with muscarinic receptors and transactivation of epidermal growth factor receptors, with the resulting proliferation of colon cancer cells (46), lend support to the involvement of cholinergic signalling in cancer.

The fall of AChE in SCC, and the subsequent increase of the available ACh, may lead to cholinergic over-stimulation in the neighbourhood cells and enhanced proliferation. The increased amount of ACh in cancerous lung pieces (Table II) agrees with the drop of AChE activity and/or overexpression of ChAT, and supports a role for cholinergic over-stimulation in lung cancer.

However, not always cholinergic stimulation triggers mitogenic actions. In fact, opposite effects in cells are often observed after nicotinic or muscarinic stimulation. In several SCLC cell lines, for instance, nicotinic activation leads to proliferating effects and muscarinic stimulation to anti-proliferating signals (47). In contrast, muscarinic activation induces cell proliferation in colon (48) and prostate (49) carcinoma cell lines.

As regarded by the molecular composition of ChEs, sedimentation profiles revealed the presence in lung of abundant amphiphilic dimers (G_2^A) and fewer AChE monomers (G_1^A) (Figure 1). The similar molecular pattern of AChE in human lung, meningioma (50) and breast (29) raises the possibility that epithelial cell-derived tissues produce the same range of AChE molecules, i.e. G_2^A and G_1^A species. Their identification in colon-derived Caco-2 cells as well as in healthy and cancerous human colorectal pieces (Montenegro,M.F., Ruiz-Espejo,F., Campoy,F.J., Muñoz-Delgado,E., Páez-de la Cadena,M, Rodríguez-Berrocal,J. and Vidal,C.J. Cancer decreases active and inactive acetylcholinesterase in human colon to a different extent; manuscript in preparation) reinforces the above contention. Lung BChE activity was distributed between G_4^H, G_4^A, G_2 and G_1 components. Although a fraction of the G_4^H molecules may come from lung infiltration with plasma, the identification of the BChE-mRNA in lung pieces (Figure 4) and the occurrence in the tissue of G_2 and G_1 forms (Figure 1), which are almost undetected in human plasma, unambiguously show that lung possesses the capacity for producing BChE. The occurrence of proline-rich membrane anchor (PRiMA)-bearing G_4^A BChE forms in healthy lung and their absence from tumours raise the possibility that the synthesis of the PRiMA structural subunit (51) is unpaired by the neoplastic process.

Regardless of the kind of cell from which tumours arise, lung malignancy does not alter the molecular composition of AChE, but the greater interaction of the lectin Con A with AChE of SCC than of AC or LCC (Figure 2) shows that the sugar composition of the enzyme in SCC is specifically modified by malignancy. The aberrant glycosylation of AChE in large airways epithelial cells, from which SCC arise, as well as in human hepatoma HuH-7 cells (52) and malignant breast (29) agrees with the reported malfunctioning of glycosyltransferases in cancerous tissues (53).

The lack of any noticeable change in the distribution of AChE forms in neoplastic lung and breast (29) does not mean that the same occurs in other tissues. In human lymph nodes, for instance, the composition of AChE variants is severely modified by breast cancer metastasis (30). The maintenance of the amphiphilic dimers and monomers (the molecules made of AChE-H subunits) in metastasized lymph nodes (30), despite the strong reduction of AChE activity and the loss of the tetrameric and asymmetric variants (the molecules made of AChE-T subunits), shows that cancer

prevents the synthesis of AChE-T subunits while the production of AChE-H subunits continues. As for the synthesis of AChE-mRNAs in neoplastic tissues concerns, it is worth mentioning the Soreq's group reports showing that the aggressiveness of astrocytoma is related with an alternative splicing shift from the AChE-T to the AChE-R-mRNA (31). The need of freshly made AChE-T protein for addressing several cell lines to apoptosis (32) makes it possible that the incapacity of cancerous tissues for making AChE-T subunits is translated into an increased number of cells.

Alternatively, assuming the existence of active and inactive AChE variants in human lung and other tissues (see later), the possibility remains that cancer affects the normal balance of active and inactive AChE molecules. If so, failures in the post-translational process required for converting the inactive into active AChE variants, rather than changes in the splicing pattern or in the amount of AChE-mRNAs, may account for the drop of AChE activity in SCC, LCC and cancerous lymph nodes (30). Whether cancer alters the relative content of the various AChE transcripts, its translation into protein and/or the conversion of inactive into active AChE is under current research.

As regarded by the western blot results (Figure 3), the labelling of 72, 68 and 50 kDa proteins with antibodies against the N-terminal peptide of human AChE and the absence of the 72 and 50 kDa proteins from the sheet blotted with antibodies against the C-terminal fragment of the AChE-T subunit allowed us to assign the 72, 68 and 50 kDa proteins to the AChE-H, AChE-T and AChE-R subunits, respectively. Although a more profound study is needed before the labelled proteins can definitively be assigned to the various AChE subunits, as a working hypothesis, it can be proposed that lung produces the three AChE subunits. The results obtained by RT–PCR, where the set of AChE-mRNAs is observed (Figure 4), support this idea.

In addition, the deeper immunolabelling of the 68 and 50 kDa proteins in the SCC than in the control lane, despite the equal units of activity loaded in them, indicates that the amount of immunolabelled AChE protein per unit of AChE activity is higher in SCC than in healthy lung, which in turn supports the presence of inactive AChE in lung. Although further studies are required for setting the structural properties of the inactive AChE molecules, their presence in SCC (and possibly in the adjacent healthy tissue) raises the possibility that the drop of AChE activity in SCC and LCC comes from an impairment in the process involved in the conversion of inactive AChE into the active form.

Summarizing, the great drop of AChE activity in SCC, the lower reduction in LCC and its maintenance in AC reveal that the change of activity in lung tumours depends on the their histopathological appearance. The distribution of AChE molecules remains unmodified in lung cancers while the amphiphilic BChE tetramers almost disappear. The raised level of ACh, owing to the fall of ChE activity in some types of lung cancer, joined to the cell proliferating effects of cholinergic agonist support a role for ChEs in lung cancer biology. The possible involvement of AChE in cell growth, proliferation and apoptosis is currently under research. The results may throw light on the linkage between the non-neuronal cholinergic system of lung and cancer, which eventually may lead to the development of new therapeutic approaches for improving patient survival and the quality of life.

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