Preliminary exploration of the expression of acetylcholinesterase in normal human T lymphocytes and leukemic Jurkat T cells

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Abstract. The classic enzymatic function of acetylcholinesterase (AChE) is the hydrolysis of acetylcholine (ACh) in the neuronal synapse. However, AChE is also present in nonneuronal cells such as lymphocytes. Various studies have proposed the participation of AChE in the development of cancer. The ACHE gene produces three mRNAs (T, H and R). AChE-T encodes amphiphilic monomers, dimers, tetramers (G_1^A, G_2^A and G_4^A) and hydrophilic tetramers (G_4^H). AChE-H encodes amphiphilic monomers and dimers (G_1^A and G_2^A). AChE-R encodes a hydrophilic monomer (G_1^H). The present study considered the differences in the mRNA expression (T, H and R) and protein levels of AChE, as well as the molecular forms of AChE, the glycosylation pattern and the enzymatic activity of AChE present in normal T lymphocytes and leukemic Jurkat E6-1 cells. The results revealed that AChE enzymatic activity was higher in normal T lymphocytes than in Jurkat cells. Normal T cells expressed AChE-H transcripts, whereas Jurkat cells expressed AChE-H and AChE-T. The molecular forms identified in normal T cells were G_2^A (5.2 S) and G_1^A (3.5 S), whereas those in Jurkat cells were G_2^A (5.2 S), G_1^A (3.5 S) and G_4^H (10.6S). AChE in Jurkat cells showed altered posttranslational maturation since a decrease in the incorporation of galactose and sialic acid into its structure was observed. In conclusion, the content and composition of AChE were altered in Jurkat cells compared with those in normal T lymphocytes. The present study opened new avenues for exploring the development of novel therapeutic strategies against T-cell leukemia and for identifying potential molecular targets for the early detection of this type of cancer.

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Introduction

The classical enzymatic function of AChE is the precise temporary hydrolysis of ACh at the cholinergic synapse. However, the biological role of AChE is not limited to cholinergic transmission (1,2). The aforementioned findings are supported by the high levels of AChE activity estimated in nonneuronal tissues, such as blood cells, erythrocytes, lymphocytes and megakaryocytes (3). The structure and functions of AChE have been the subject of various investigations worldwide. However, a number of unresolved questions remain, both in terms of its mechanism of action, synthesis processes and transport through cellular compartments. The physiological meaning of AChE molecular polymorphisms is also unknown, although their roles in apoptosis, proliferation, cell differentiation, cell adhesion, neurogenesis and hematopoiesis and possible participation in other biological activities have begun to be elucidated (1,4-8).

The structural complexity of human AChE is due to a gene with a size of 7 kb located in the q22 region of chromosome 7 (9,10). The ACHE gene produces three different mRNAs (T, H and R) through an alternative splicing process (11). The generated mRNAs share exons E2, E3 and E4, which encode the catalytic domain of AChE (12,13). The AChE-T-type mRNA is assembled with exons E2-E3-E4 and E6; it encodes

amphiphilic monomers, dimers and tetramers (G_1^A, G_2^A and G_4^A), hydrophilic tetramers (G_4^H) and hetero-oligomeric associations with a collagen-like stem Q, structurally conforming to the asymmetric forms (A_4, A_8 and A_{12}, which contain one, two and three tetramers, respectively), or with the transmembrane protein proline-rich membrane anchor (PRiMA), constituting membrane-bound tetramers PRiMA-G_4^A (14-16). The AChE-H-type mRNA contains exons E2-E3-E4-E5, which encode Type I amphiphilic monomers and dimers (G_1^A and G_2^A) in which each subunit has a covalently linked glycophosphatidylinositol residue (11,16,17). The AChE-R-type mRNA, also called read-through, does not undergo splicing after the last exon encoding the catalytic domain and, therefore, has the arrangement E2-E3-E4-I4-E5. The C-terminus of the R subunit consists of 30 amino acids and lacks Cys and thus the subunits remain as hydrophilic monomers (G_1^H) (18,19). Abnormalities in the ACHE gene have been observed in various types of cancer, such as ovarian, breast, prostate, liver and some types of myeloid leukemia (5,20-22).

In general, both brain and nonbrain tumors present an alteration in AChE activity; in a number of cases, a decrease in specific AChE activity is observed (23,24). Lung cancer shows a decrease in AChE-T mRNA levels (25), whereas colon cancer shows decreases in AChE-T, AChE-H and AChE-R mRNA levels (26,27). The decrease in mRNA levels is related to a decrease in AChE activity (25,28). Furthermore, alterations in the ACHE gene in breast cancer decrease AChE enzymatic activity in axillary lymph nodes (29). In other types of cancer, such as in gliomas (30), liver tumors (5,31), gastric cancer (32) and head and neck carcinomas, a reduction in the enzymatic activity of AChE has been observed, where the low activity of AChE is associated with worse survival (33,34). In patients with prostate cancer, a decrease in AChE activity in the blood has been reported compared with that in a control group of healthy subjects; this finding opens the possibility of proposing AChE as a cancer biomarker (35). All these reported events, where a decrease in both the expression and enzymatic activity of AChE occur, cause an increase in the amount of ACh that leads to cholinergic overstimulation and an increase in cell proliferation (36-40).

Antisense polynucleotides against AChE produce essential changes in hematopoiesis, decreasing apoptosis and increasing the proliferation of blood cells (41,42). Organophosphate pesticides are potent inhibitors of AChE and their use in agricultural activities is associated with the development of non-Hodgkin lymphoma and leukemia (21,43-46). Other studies of rat mammary glands have demonstrated that the inhibition of AChE with physostigmine induces cancer development, revealing a relationship between a decrease in AChE activity and carcinogenesis (47).

A clear relationship exists between low AChE activity and a poor prognosis for cancer patients. However, determining why reducing AChE activity and protein content induces tumor progression and aggressiveness is important. With respect to the information available regarding AChE gene and protein expression in cancerous tissues, research still needs to be performed to clarify the molecular mechanisms by which AChE could participate in types of cancer. Exploring study models that reveal new information about the relationship of this enzyme with the processes of altered cell proliferation is essential (5). Based on the aforementioned findings, hematopoietic cells, mainly T lymphocytes, have a well-established nonneuronal cholinergic system called the lymphocytic cholinergic system (3,48-50), which is why the present study proposed it as a study model to obtain an improved understanding of the differences in AChE expression between normal cells and cancer cells.

Based on the reported evidence that AChE expression is altered in cancer cells compared with that in their normal counterparts, the present study aimed to compare AChE expression at the mRNA and protein levels, as well as the glycosylation and enzymatic activity of the AChE protein between normal T lymphocytes and the human T lymphoblast cell line Jurkat E6-1.

Materials and methods

Reagents and general materials. Fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine and sodium pyruvate solutions were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). The other reagents mentioned were acquired from MilliporeSigma. ethylenediaminetetraacetic acid (EDTA)-Vacutainer tubes for blood collection from healthy volunteers were acquired from Becton, Dickinson and Company.

Cell culture. The human T lymphoblast cell line Jurkat E6-1 (TIB-152) was obtained from the American Type Culture Collection. Jurkat E6-1 cells were cultured in RPMI-1640 medium (MilliporeSigma) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin. Jurkat cells from passages 2 to 5 were grown at 37˚C in a humidified 5% CO_2 atmosphere for all experiments.

The human liver cancer cell line HepG2 (HB-8065) was obtained from the American Type Culture Collection. HepG2 cells were cultured in DMEM (MilliporeSigma) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

Obtaining T lymphocytes from healthy adults. The use of lymphocytes from healthy donors was included to perform comparative tests with leukemic cells. The sample consisted of 10 young adults (aged 18-22 years) selected from the period between January, 2023 and April, 2023, according to the following inclusion and exclusion criteria.

The inclusion criteria were: Age between 18-22 years, weight between 50-80 kg, systolic blood pressure between 90-160 mm Hg, diastolic blood pressure between 60-90 mm Hg, pulse between 50-100 beats and fasting for 6-8 h. The volunteers could not consume foods with fat, eggs, milk, or their derivatives 24 h before donation.

The exclusion criteria were as follows: Pregnant or breastfeeding women; individuals who had suffered from flu, cough, diarrhea or dental infection in the last 14 days; individuals who had taken medications in the last five days; individuals who had undergone endodontic treatment, acupuncture or had tattoos or piercings in the last 12 months; those who had undergone surgery in the last six months; those who had been vaccinated in the last 30 days; and those who consumed alcoholic beverages in the 72 h prior to donation.

Informed consent was obtained from all individual participants included in the study to the collection and use of their cells strictly for research purposes. The research ethics committee approved the use of blood samples donated by healthy adults with a registration in the National Bioethics Commission with the number CONBIOETICA-09-CEI-025-20161215 of the National Institute of Pediatrics, Mexico.

Density gradient separation was performed from 20 ml of blood, which was generated via density gradient separation using Lymphoprep density gradient medium (STEMCELL Technologies) and centrifuged at 800 x g for 20 min at room temperature (20-25˚C) to extract the mononuclear cells (including T lymphocytes), which were subsequently resuspended in 1 ml of PBS.

Magnetic beads conjugated with an anti-CD3 antibody (specific T lymphocyte marker) MAC (Miltenyi Biotec, Inc.) were added to the suspension of mononuclear cells. T lymphocytes were isolated by magnetic separation with MiniMACS Starting Kit (Miltenyi Biotec, Inc.) according to the manufacturer's protocol. This device collects the entire cell suspension and separates the cells into a positive fraction (T lymphocytes) and a negative fraction (the remainder of the cells). The magnetic field of the MACS column allows for minimal labeling of target cells with small microbeads. This means that there are plenty of surface epitopes available for fluorescent staining and flow cytometry analysis. The positive fraction was recovered and the purity of the T lymphocytes was determined via flow cytometry with a FACSCalibur 2 Laser, 4 Color flow cytometer (Becton, Dickinson and Company); the CD3-PerCP antibody was used incubated for 30 min at room temperature (20-25˚C). The samples were processed with CellQuest Pro v 5.2.1. Software (BD Biosciences) via the SSC and the association with CD3-PerCP.

Trypan blue (MicroLab-Mexico) was used to determine the viability and density of total leukocytes, purified T lymphocytes and Jurkat cells. Cells were incubated with equal volume of 0.4% trypan blue solution for 3 min at room temperature (20-25˚C). Viable cells at a density of $1x10^7$ cells/ml were used for the assays.

Total RNA extraction. Total RNA was extracted from $10x10^7$ cell samples with 1 ml of TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The isolated RNA was partially dissolved in 100 μl of RNase-free water and incubated at 55˚C for 5 min. The RNA concentration and purity were determined with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.) by calculating the optical density ratio at wavelengths of 260/280 nm and 260/230 nm.

Reverse transcription (RT)-PCR amplification. The RT-PCR process was performed using the one-step RT-PCR kit (Qiagen, Inc.) and the following primer pairs: AChE-H (487 bp), forward 5'-TCTCGAAACTACACGGCAGA-3' and reverse 5'-TGAGGAGGAAGGGAGCACTA-3'; AChE-T (444 bp), forward 5'-TCTCGAAACTACACGGCAGA-3' and reverse 5'-GCCCAGCCCTGAAATAAATAG-3'; AChE-R (333 bp), forward 5'-TCTCGAAACTACACGGCAGA-3' and reverse 5'-GGGGAGAAGAGAGGGGTTAC-3'; and β-actin (500 bp) as a housekeeping gene, forward 5'-CACTGGCATCGTGATGGACT-3' and reverse 5'-AATGCCAGGGTACATGGTGG-3'. A total of 1 μM of each primer, 1 μl of a 40 mM dNTP mixture, 5 μl of 5X Q solution, 1 μl of enzyme mix and 1.5 μg of RNA sample were used (titrations were performed using different amounts of RNA, 0.5-2.0 μg), for a final volume of 25 μl that was completed with RNase-free water.

The samples were placed in a Mastercycler Gradient Thermal Cycler (Eppendorf SE) and cycled with the following program: 50˚C for 30 min and 95˚C for 15 min for reverse transcription, followed by 39 cycles (1 min at 94˚C, 1 min at 50˚C and 1 min at 72˚C) for amplification. After the final cycle, the temperature was maintained at 72˚C for 10 min. The amplified DNA fragments were visualized on a 2% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide. Image acquisition was performed using a Molecular Imager Gel Doc XR+system (Bio-Rad Laboratories, Inc.). The optical density of each band was calculated after background subtraction and normalization to β-actin for semiquantitative analysis using Image Studio 4.0 software (LI-COR Biosciences).

Solubilization of the AChE protein. For AChE solubilization from isolated T lymphocytes and Jurkat cells, $1x10^7$ cells were used per sample. Each sample was homogenized with HEPES saline buffer [15 mM HEPES, 1 M NaCl, 50 mM $MgCl_2$, 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid and 3 mM EDTA, pH 7.5] with a mixture of protease inhibitors at 10% cell weight/buffer volume (w/v). The homogenate was centrifuged at 200,000 x g in a Beckman Ti 60 rotor (Beckman Coulter, Inc.) for 1 h at 4˚C to separate soluble AChE from AChE weakly bound to the membranes. After the first supernatants (S_1) were collected, the precipitates were homogenized with HEPES saline buffer supplemented with 1% (w/v) Triton X-100. These suspensions were subsequently centrifuged at 200,000 x g for 1 h at 4˚C to obtain the enzyme bound to the membranes in the second supernatant (S_2). Centrifugation was performed using a Beckman XL-90 Optima centrifuge (Beckman Coulter, Inc.). The enzymatic activity of AChE was subsequently determined in both fractions (S_1 and S_2).

Estimation of AChE enzymatic activity. The measurement of AChE activity was performed via spectrophotometry with a Cary 50 spectrophotometer (Agilent Technologies, Inc.) using the Ellman method (51). The reaction medium contained 100 mM phosphate buffer, pH 8.0, with 0.33 mM 5,5'-dithiobis-(2-nitrobenzoic) acid, 1 mM acetylthiocholine iodide and 50 μM Iso-OMPA, butyrylcholinesterase inhibitor. One AChE unit (U) represents the enzyme that hydrolyzes 1 μmol of substrate per min at 37˚C.

AChE activity in the fractions collected from the sucrose gradients was determined using the Ellman method adapted to a microassay method using a microplate spectrophotometer Epoch (BioTek; Agilent Technologies, Inc.), for which transparent plastic plates were used (Nunc), with 96 wells of 400 μl and a flat bottom. Enzymatic activity was expressed in arbitrary units (AUs) such that one AU represented an increase in A405 of 0.001 per min and for each μl of the sample at room temperature. The Bradford method was used to determine the protein content with bovine serum albumin as a standard protein (52).

Profile of the molecular forms of the AChE protein. The molecular forms of AChE were determined by gradient centrifugation using two sucrose solutions of different concentrations (5 and 20% w/v) prepared in 10 mM Tris-HCl buffer, pH 7.0, with 1 M NaCl, 50 mM $MgCl_2$ and Triton X-100 (0.5%, W/V). The proteins used as sedimentation standards were bovine liver catalase (11.4S) and bovine intestine phosphatase (6.1S). The gradients were centrifuged at 200,000 x g for 18 h at 4˚C in an SW41Ti tilting rotor (Beckman Coulter, Inc.). Following centrifugation, 37-40 fractions were collected using the peristaltic pump Econo Gradient Pump and a fraction collector model 2110 (both from Bio-Rad Laboratories, Inc.). Sedimentation coefficients were determined according to Martin and Ames's method (53), comparing the distance traveled by the AChE protein with that of standard proteins.

AChE protein glycosylation profile. Lectins from *Concanavalia ensiformis* (ConA), *Lens culinaris* (LCA), *Triticum vulgaris* (WGA) and *Ricinus communis* (RCA) were used to determine possible differences in the binding of AChE from T lymphocytes and Jurkat cells to glycans. ConA recognizes mannose residues, LCA recognizes D-mannose bound to fucosylation centers, WGA recognizes N-acetyl-glucosamine and RCA recognizes galactose or sialic acid.

Aliquots of the mixture of S_1+S_2 extracts (0.5 ml) of T lymphocytes and Jurkat cells were incubated with 0.25 ml of Sepharose-4B (nonspecific binding enzyme control) or ConA, LCA, RCA or WGA overnight at 4˚C with constant stirring. AChE activity in the supernatants was assessed via the microassay method to calculate the percentage of protein interacting with lectin and the supernatant corresponding to Sepharose-4B, which represented 100% of the non-retained proteins, was used as a reference.

Statistical analysis. The values of the means and variances for solubilized enzyme activity, protein content, interaction with lectins and the proportion of each molecular form were obtained for normal lymphocytes and leukemic cells for statistical analysis. The results were compared using the Mann-Whitney U test to establish a significant difference. Data were analyzed using NCSS 2007 Statistical Software (NCSS, LLC). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Collection of T lymphocytes and analysis of purity using flow cytometry. The average number of leukocytes isolated from 10 blood samples of 20 ml was $7.13x10^7$±0.89. The average number of T lymphocytes isolated from the total leukocyte population by magnetic separation was $1.02x10^7$±1.12 and their viability was 100% (Table I). Fig. 1 shows the magnetic separation of T lymphocytes by flow cytometry. Of the total leukocytes, ~25% were positive for the CD3 surface marker, indicating the presence of T lymphocytes. The average percentage of the 10 samples analyzed using flow cytometry was 98.36%, indicating that the cell purification process provided high performance.

Jurkat cells were also labeled with CD3-PerCP to determine the percentage of $CD3^+$ cells. The results revealed that 84.89% of these cells were positive for the CD3 surface marker. Similarly, Fig. 1C shows an analysis performed using flow cytometry on a sample of Jurkat cells labeled with CD3-PerCP.

Table I. Viability and density of total leukocytes and purified T lymphocytes.

Source	Cell number/ml (n=10)	Viability (n=10)
Leukocytes	$7.13x10^7$±0.89	100%
T lymphocytes	$1.02x10^7$±1.12	100%

Peripheral blood samples of 20 ml each were obtained from 10 healthy adults. Total leukocytes were obtained by density gradient separation with Lymphoprep and T lymphocytes were purified by magnetic separation using an anti-CD3 antibody conjugated to magnetic beads. Cell viability and density were determined using Trypan blue. The table shows the averages and standard deviations of the number of cells/ml and the percentage of cell viability obtained at each stage of cell separation.

One-step RT-PCR of the ACHE mRNA. Normal human T lymphocytes were used for mRNA titration with different primers. The results revealed that the optimal concentration for amplification assays was 1.5 μg of RNA.

The detection of AChE mRNA variants in normal T cells revealed only one amplified product, corresponding to the AChE-H transcript; the products of the amplifications revealed a band in the gel of ~487 bp (Fig. 2). These results indicated that human T lymphocytes express only the AChE-H transcript, which encodes membrane-anchored amphiphilic dimers (17,54).

In the analysis of the expression of the ACHE gene transcripts in Jurkat leukemia cells, the results revealed that the transcripts detected corresponded to AChE-H (487 bp) and AChE-T (444 bp); the latter was not detected in normal T lymphocytes (Fig. 3). With these data, a comparative analysis of the expression of AChE in T lymphocytes and leukemic cells could be later conducted. Fig. 4 shows the amplification products of the alternative AChE transcripts detected in normal T cells and Jurkat cells; as a positive control for AChE-R, a 574 bp product was obtained using the same set of primers for AChE-H and mRNA extracted from HepG2 cells.

Finally, densitometry analyses were performed to obtain the intensity of each band, thus obtaining an average of the intensity of the β-actin control, which facilitated establishing the relative intensity index of each band of the amplicons corresponding to AChE in each sample (Table II).

Estimation of AChE activity in normal T lymphocytes and Jurkat cells. The AChE activity in the enzyme extracts of T lymphocytes solubilized by ionic force (S_1) or detergent (S_2) did not change. These results indicated that in human T lymphocytes, a similar proportion of AChE is weakly bound and that AChE is strongly bound to the membrane.

On the other hand, the results obtained from the solubilization of the AChE protein in Jurkat cells revealed higher AChE activity in the weakly membrane-bound fraction

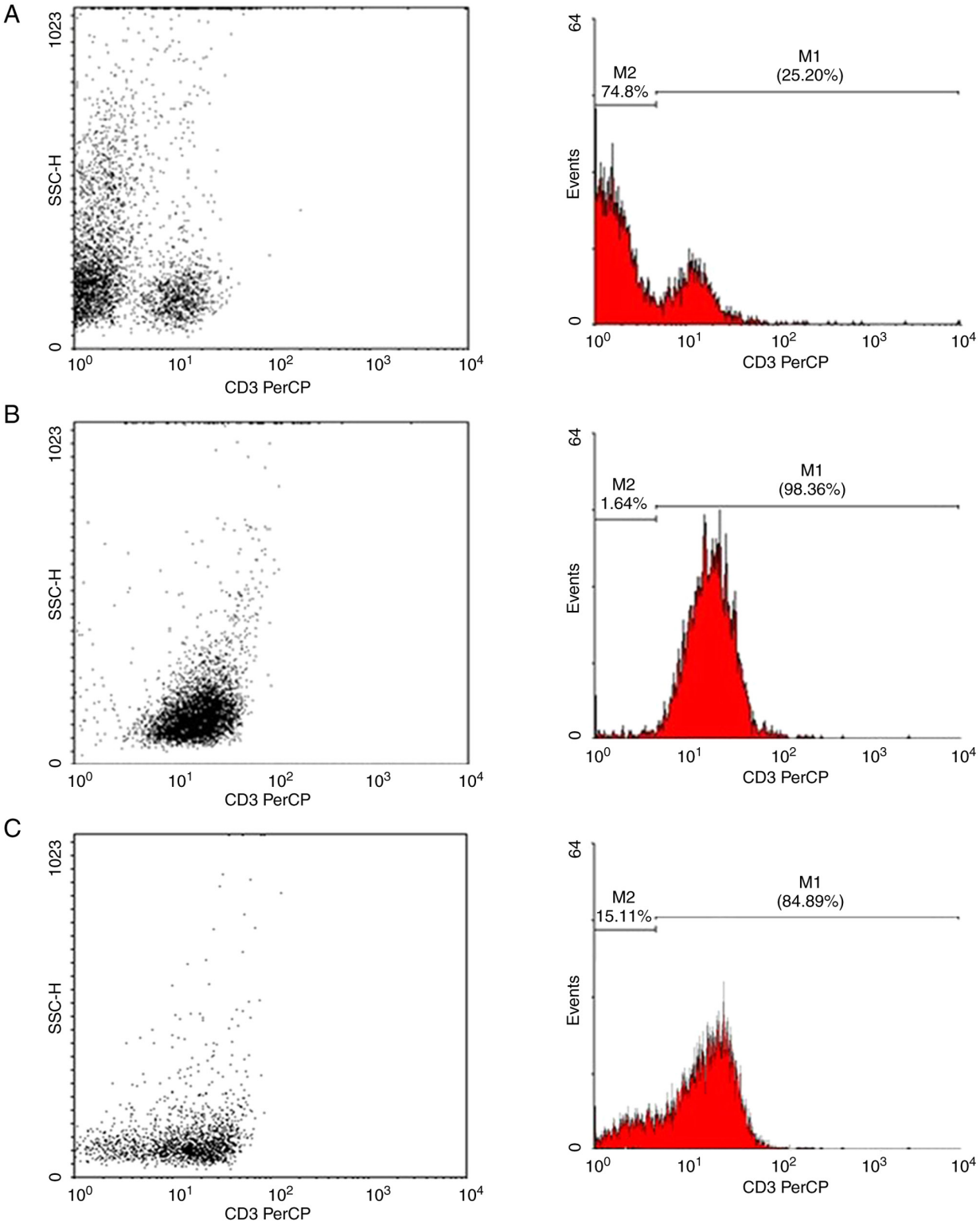

Figure 1. Flow cytometry analysis of T lymphocytes identified by the CD3 surface marker. Total leukocytes were obtained using density gradient separation with Lymphoprep and then resuspended in 1 ml of PBS. Magnetic nanoparticles conjugated with anti-CD3 were used to purify T lymphocytes using AUTOMACS equipment from Miltenyi Biotec, Inc. Total leukocytes, purified T lymphocytes and Jurkat cells were incubated with an anti-CD3 monoclonal antibody conjugated with the PerCP fluorochrome to detect the CD3 surface marker. (A) Prior to magnetic separation, 25.2% of the total leukocytes were found to be positive for the CD3 marker. (B) Following purification, the average purity of the T lymphocytes was 98.36%. (C) Jurkat cells labeled with a PerCP-conjugated anti-CD3 antibody were 84.89% positive for the CD3 surface marker.

(S_1) than in the strongly membrane-bound fraction (S_2). Table III shows the protein content, AChE activity and AChE-specific activity obtained from the S_1 and S_2 extracts of normal T lymphocytes and Jurkat cells. Specifically, Jurkat cells had lower activity than normal T lymphocytes. These data were thought-provoking as, although the Jurkat cells expressed a transcript, their activity levels decreased.

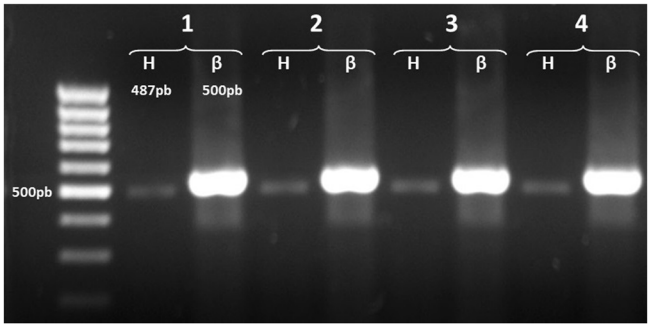

Figure 2. One-step RT-PCR analysis of ACHE mRNA transcripts in normal T lymphocytes. Total RNA isolated from T lymphocytes of four healthy donors was used for one-step RT-PCR with specific primers for the AChE-H (487 bp), AChE-T (444 bp) and AChE-R (333 bp) variants, along with β-actin (500 bp) as a housekeeping gene control. The reaction was performed with 1.5 μg of RNA, 1 μM each primer and the One-Step RT-PCR Kit (Qiagen GmbH). The PCR products were separated on a 2% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide and visualized under UV light. A single amplified product corresponding to the AChE-H variant (487 bp) was detected in each sample, along with the β-actin control (500 bp). RT-PCR, reverse transcription PCR; AChE, acetylcholinesterase.

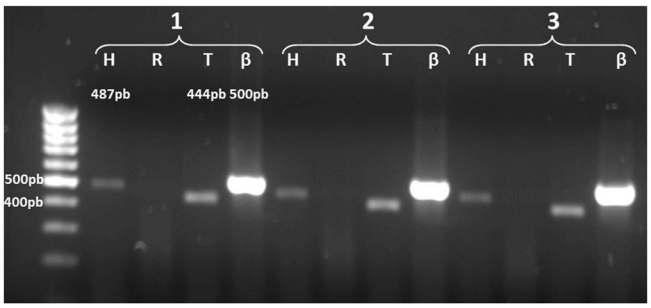

Figure 3. One-step RT-PCR analysis of ACHE mRNA transcripts in Jurkat E6-1 leukemia cells. The total RNA isolated from Jurkat E6-1 leukemia cells was used for one-step RT-PCR with specific primers for AChE-H (487 bp), AChE-T (444 bp) and AChE-R (333 bp) variants, along with β-actin (500 bp) as a housekeeping gene control. The PCR products were separated on a 2% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide and visualized under UV light. The analysis revealed the presence of AChE-H (487 bp) and AChE-T (444 bp) transcripts, whereas AChE-R was not detected. RT-PCR, reverse transcription PCR; AChE, acetylcholinesterase.

Glycosylation profile of the AChE protein in T lymphocytes and Jurkat cells. An analysis of the interaction with lectins revealed the posttranslational maturation process that glycoproteins undergo. In the case of T lymphocytes, the AChE protein had a weak interaction with the ConA lectin (33%), the interaction with LCA was 66%, the interaction level observed with WGA was 63% and the interaction with RCA was 75% (Fig. 5). Meanwhile, the glycosylation profile of the AChE protein differed in Jurkat leukemic cells. The interactions of the AChE protein with the ConA, LCA, WGA and RCA lectins were 66, 72, 67 and 67%, respectively. The difference in the level of AChE glycosylation between T lymphocytes and leukemic T cells indicated that the posttranslational maturation process of AChE is modified in the neoplastic state.

Profile of the molecular forms of AChE in T lymphocytes and Jurkat cells. The results for the molecular forms of AChE with sedimentation coefficients of 5.2S and 3.5S were

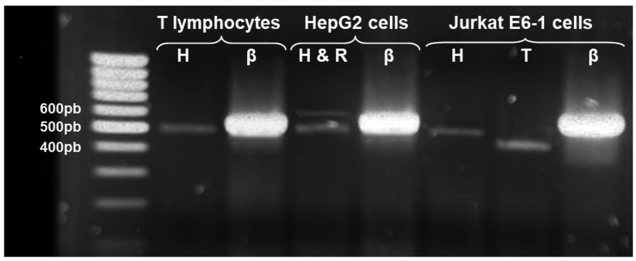

Figure 4. One-step RT-PCR analysis of ACHE mRNA transcripts in human T lymphocytes and cell lines. Total RNA isolated from normal human T lymphocytes, HepG2 hepatoma cells and Jurkat E6-1 leukemia cells and was used for one-step RT-PCR. Specific primers were used for AChE-H (487 bp), AChE-T (444 bp) and AChE-R (574 bp) variants, along with β-actin (500 bp) as a housekeeping gene control. The PCR products were separated on a 2% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide and visualized under UV light. Normal T lymphocytes expressed only the AChE-H transcript (487 bp), whereas Jurkat cells expressed both AChE-H (487 bp) and AChE-T (444 bp) transcripts. RNA from the HepG2 cell line was used as a positive control for AChE-R amplification, resulting in a band at 574 bp with the same primer set used for AChE-H (487 bp). β-Actin bands for each cell line were included for normalization purposes. RT-PCR, reverse transcription PCR; AChE, acetylcholinesterase.

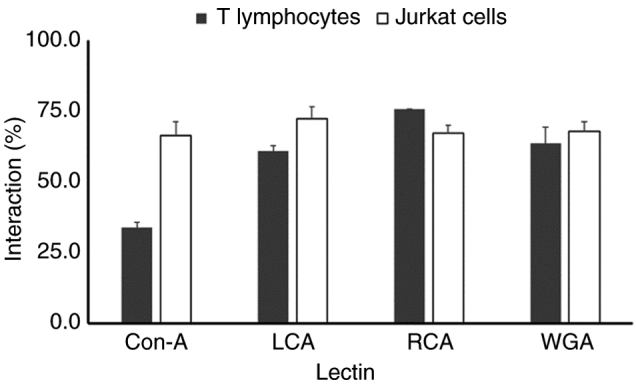

Figure 5. Lectin affinity chromatography analysis of AChE glycosylation in human T lymphocytes and Jurkat cells. Solubilized AChE protein extracts (S_1+S_2 fractions) from normal human T lymphocytes and Jurkat E6-1 leukemia cells were incubated overnight at 4˚C with Sepharose-4B beads (control for nonspecific binding) or lectin-conjugated Sepharose-4B beads specific for ConA, recognizing mannose residues, LCA recognizing D-mannose bound to fucose residues, RCA, recognizing galactose or terminal sialic acid residues and WGA recognizing N-acetyl-glucosamine residues. Unbound AChE activity in the supernatants was measured via a microplate assay and expressed as a percentage of the total AChE activity in the Sepharose-4B control (100%). T lymphocytes: AChE displayed decreased binding to ConA (33%), moderate binding to LCA (66%) and WGA (63%) and increased binding to RCA (75%). Jurkat cells: AChE exhibited increased binding to ConA (66%) and LCA (72%) compared with T-lymphocytes, suggesting increased levels of mannose and fucose-associated mannose residues. Conversely, Jurkat cells showed decreased binding to RCA (67%) compared with T lymphocytes, indicating potentially reduced levels of galactose or sialic acid residues. Binding to WGA (67%) remained statistically similar ($P<0.05$) between both cell lines. AChE, acetylcholinesterase; ConA, Concanavalin A; LCA, *Lens culinaris* agglutinin; RCA, *Ricinus communis* agglutinin; WGA, wheat germ agglutinin.

consistent with previously reported values for amphiphilic dimers (G_2^A, 5.2S) and amphiphilic monomers (G_1^A, 3.5S) found in T-cell extracts (55,56). In the sedimentation analysis of the Jurkat cell extracts, amphiphilic dimers (G_2^A, 5.2S), amphiphilic monomers (G_1^A, 3.5S) and hydrophilic tetramers (G_4^H, 10.6) were the AChE molecular forms identified. The

Table II. Relative intensity of mRNA splicing isoforms for the ACHE gene.

	Expressed AChE transcript		
Cell type	H	R	T
Normal T lymphocytes	0.2326±0.0102	ND	ND
Jurkat E6-1 cells	0.1909±0.0050	ND	0.4638±0.0058
HepG2 cells	0.2615±0.0308	0.0657±0.0128	-

The values represent the means and respective standard deviations of four data points obtained from the densitometry analysis of samples of normal T lymphocytes (n=4), Jurkat cells (n=3) and HepG2 cells (n=3); the last was used as a positive control for the expression of the AChE-R transcript. The reverse transcription PCR data were normalized to the control of the β-actin gene expressed in each cell type, with a mean value of 206.39±3.5756. The expression of the AChE-H transcript was significantly higher in normal T cells than in Jurkat cells (Mann-Whitney U test; $P<0.05$). AChE, acetylcholinesterase.

Table III. Solubilized acetylcholinesterase activity of normal T lymphocytes and Jurkat cells.

	Protein (mg/ml)	AChE (U/ml)	A.E. AChE (U/mg)
T-lymphocytes			
Fraction S_1	1.96±0.0035	0.216±0.0849	0.1101±0.0433
Fraction S_2	2.22±0.0049	0.294±0.0255	0.1323±0.0115
Jurkat Cells			
Fraction S_1	4.86±0.0045	0.287±0.0312	0.0590±0.0064
Fraction S_2	2.50±0.0164	0.088±0.0000	0.0353±0.0000

Means and standard deviations of the protein content, AChE activity by volume and A.E. estimated from solubilization of T-lymphocyte (n=3) and Jurkat cell (n=3) samples. Fraction S_1 was saline HEPES buffer and S_2 was saline HEPES buffer containing Triton X-100. The AChE activity in the S_1 and S_2 fractions of T lymphocytes was similar (Mann-Whitney U test, $P\leq0.05$). In the case of Jurkat cells, the AChE activity in the S_1 fraction was higher than that in the S_2 fraction. The A.E. of AChE in the S_1 and S_2 fractions of Jurkat cells was lower than that in normal T cells. A.E., specific activity; AChE, acetylcholinesterase.

latter is encoded by the AChE-T transcript, thus confirming the presence of this alternative splicing variant in the studies conducted via RT-PCR (57). Fig. 6 shows the density gradient and the molecular forms of AChE present in normal T lymphocytes and Jurkat cells.

Discussion

The expression of the ACHE gene in normal T lymphocytes and Jurkat E6-1 leukemic cells was analyzed using RT-PCR. AChE-H transcripts were detected in the first cell type, but AChE-T or AChE-R transcripts were not. These results agreed with a previous study of peripheral blood cells, including lymphocytes and erythrocytes, where the exclusive presence of the AChE-H transcript has been reported. This transcript encodes the AChE molecular forms of amphiphilic dimers and monomers (G_2^A and G_1^A) anchored to the plasma membrane by a glycosylphosphatidylinositol bond (17).

By contrast, the analysis of AChE transcripts in Jurkat leukemic cells using RT-PCR allowed the detection of AChE-H transcripts and the AChE-T variant, which demonstrated an alteration in the expression of the AChE gene in this cell line. This finding coincided with the results observed by Liao *et al* (58) regarding the expression of these transcripts in the Jurkat line.

In a previous study of P19 semidifferentiated neuronal cells via microarrays, overexpression of AChE-T transcripts was observed, which is associated with high expression levels of antiapoptotic genes and genes related to the splicing process (59). In Jurkat cells, the AChE-T transcript is probably involved in cell proliferation by regulating the expression of antiapoptotic agents and allowing the cell to survive.

The data obtained in the present study revealed that Jurkat cells presented a notable decrease in AChE activity compared with that of normal T lymphocytes. Other studies have reported lower AChE activity in cancer cells compared with normal cells (5,26,29). In the case of lung tumors, AChE activity decreases with increasing malignancy, which is related to an increase in ACh levels in these cells (25). The increase in AChE activity may be related to the development of hematological diseases and the regulation of immune function (35).

The function of lymphocytes is regulated not only by the cytokine system but also by the lymphoid cholinergic system independent of cholinergic neurons (3). A number of the components found in the nervous system, including ACh, choline acetyltransferase (ChAT), high-affinity choline transporter, muscarinic and nicotinic ACh receptors (mAChR and nAChR, respectively) and AChE, which together are known as the nonneuronal cholinergic system (NNCS), are

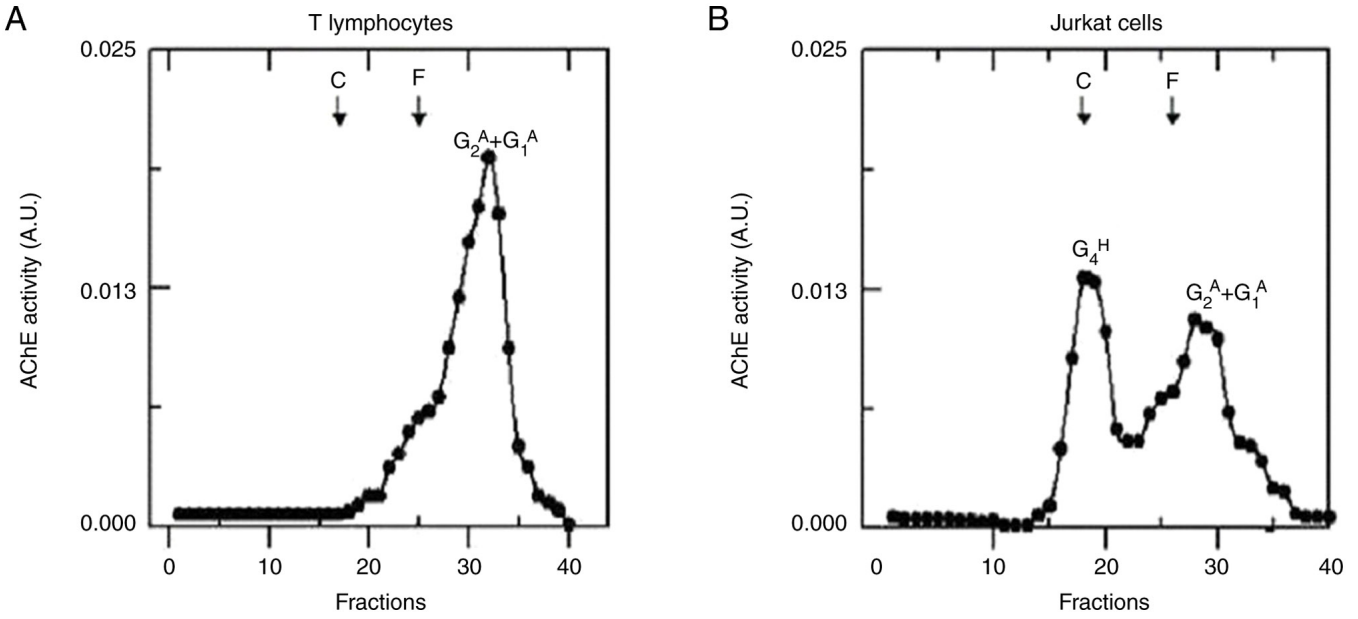

Figure 6. Sucrose density gradient sedimentation analysis of the AChE molecular forms in human T lymphocytes and Jurkat cells. The molecular forms of AChE were identified using gradient centrifugation with 5 and 20% sucrose solutions in Tris-HCl buffer containing NaCl, $MgCl_2$ and Triton X-100. Bovine liver catalase (11.4S) and bovine intestine phosphatase (6.1S) served as sedimentation standards. The gradients were subsequently centrifuged at 200,000 x g for 18 h at 4˚C. Fractions 37-40 were collected following centrifugation and sedimentation coefficients were calculated from the distance traveled by AChE compared with that traveled by standard proteins. The following marker proteins were used to determine sedimentation coefficients: Alkaline phosphatase (6.1S, F) and catalase (11.4S, C). (A) AChE protein fractions of T lymphocytes displayed sedimentation coefficients corresponding to the amphiphilic dimer G_2^A (5.2S) and the amphiphilic monomer G_1^A (3.5S). (B) AChE protein fractions of Jurkat cells revealed the presence of all three forms identified in T lymphocytes (G_2^A, 5.2S and G_1^A, 3.5S) along with an additional higher-molecular-weight form consistent with hydrophilic tetramers G_4^H (10.6S). The presence of G_4^H suggested the expression of the AChE-T transcript variant in Jurkat cells. AChE, acetylcholinesterase; A.U., arbitrary units.

expressed in lymphocytes (49,50,60). On the other hand, ACh and agonists of mAChRs and nAChRs have been reported to induce a variety of effects on lymphocytes, such as increases in the fluidity of the membrane, the synthesis of inositol triphosphate, the expression of the c-fos gene, the activation of DNA and RNA synthesis and cell proliferation. These findings support the hypothesis that ACh may regulate the functions of T lymphocytes (49,50,60).

A decrease in AChE activity can lead to an increase in the content of ACh present in the cell when it is not hydrolyzed. In the case of leukemic cells, the present study found that the specific activity of AChE was much lower than that of normal T lymphocytes. Similar results have been reported by Fujii *et al* (60), who documented that the levels of ACh in T lymphocytes are much lower than those in Jurkat cells. Thus, an increase in ACh levels in leukemic cells, produced by a lack of AChE activity, leads to an overregulation of the functions controlled by the NNCS, including the activation of cell proliferation (3). Fig. 7 shows a model that attempts to explain the induction of cell proliferation by ACh levels.

The sedimentation analysis of the molecular forms of AChE in T lymphocytes revealed the presence of a molecular structure corresponding to amphiphilic dimers and monomers (G_2^A and G_1^A), which are anchored to the membrane by GFI and originate through the expression of AChE-H transcripts (17,57,60). This finding is evidence of the expression of the AChE-H transcript detected in this type of cell. On the other hand, the sedimentation analysis of the AChE molecular forms in Jurkat cells revealed the presence of amphiphilic monomers and dimers (G_2^A and G_1^A) and hydrophilic tetramers (G_4^H). The molecular forms found in Jurkat cells were confirmed by the presence of the AChE-H transcripts responsible for the synthesis of the AChE G_2^A and G_1^A forms, as well as the expression of AChE-T transcripts from which the molecular form of AChE G_4^H is derived (57).

In this context, AChE activity has been proposed as a generic marker for small extracellular vesicles or exosomes. In Jurkat cells incubated with an extra depleted medium that can release AChE protein, a hydrophilic variant is released into the extracellular medium (58). AChE is an export protein whose synthesis is restarted in the rough endoplasmic reticulum, after which it is transported to the Golgi apparatus for modification and then secreted or deposited on the cell membrane (61).

Glycosylation variations were detected between both cell types, which suggested an alteration in the expression of AChE in a transformed cell compared with a normal cell. The primary sequence of the AChE protein contains three potential glycosylation sites linked to asparagine (Asn) residues 265, 350 and 464 through the glycosylation signal Asn-X-Ser (62). In mammals, these sites are conserved in all sequenced cholinesterases (63). When all three N-glycosylation signals of human AChE were suppressed by site-directed mutagenesis, all the sites were shown to be essential for effective biosynthesis and secretion. The extracellular AChE levels of mutants defective at one, two, or all three sites were 20-30%, 2-4% and ~0.5% of the wild-type level, respectively (62).

In a number of pathological states, the degree of AChE alteration includes posttranslational maturation, as evidenced by the modification of the glycosylation pattern of the AChE protein (25,29,64,65). A different glycosylation pattern of

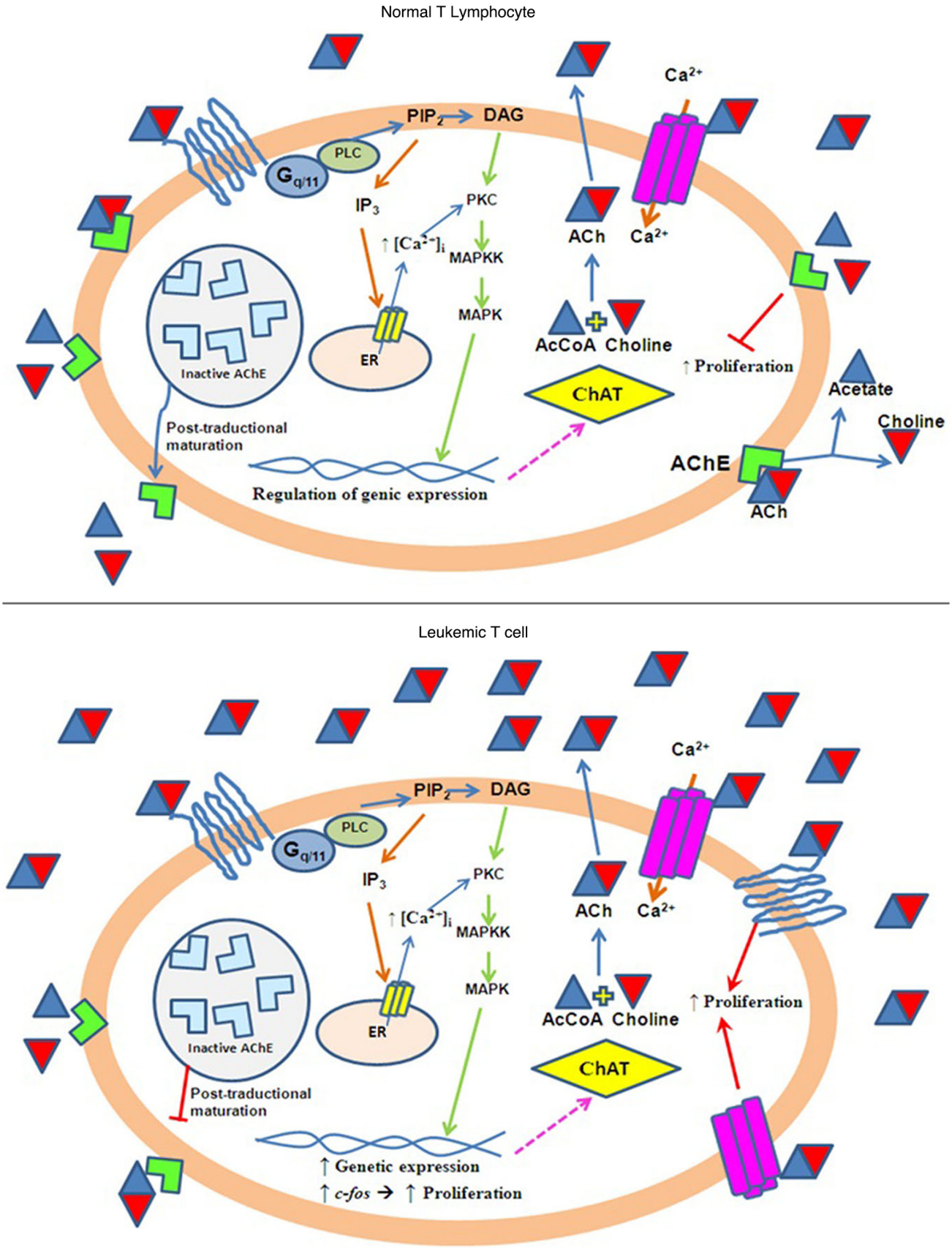

Figure 7. Schematic model of ACh-mediated induction of cell proliferation in Jurkat leukemia cells compared with normal cells. *Normal cells*: Functional AChE maintains steady-state levels of ACh via hydrolysis. ACh interacts nAChRs and mAChRs receptors at physiological levels, leading to balanced regulation of intracellular signaling pathways. Jurkat cells: Deficient AChE activity leads to increased ACh accumulation. Elevated ACh levels overstimulate nAChRs and mAChRs, resulting in the excessive activation of ChAT and PLC. PLC activation increases the Ca^{2+} release from the endoplasmic reticulum. Increased Ca^{2+} signaling promotes the expression of the proto-oncogene c-fos, which upregulates genes involved in cell proliferation. ACh, acetylcholine; AChE, acetylcholinesterase; nAChRs, nicotinic ACh receptor; mAChRs, muscarinic ACh receptor; ChAT, choline acetyltransferase; PLC, phospholipase C; PIP_2, phosphatidylinositol bisphosphate; DAG, diacylglycerol; PKC, protein kinase C; IP_3, inositol triphosphate.

the AChE proteins contained in the S_1+S_2 extracts of normal T lymphocytes and Jurkat cells was observed when they interacted with lectins of different specificities. This finding indicated that the incorporation and/or removal of sugar residues during AChE maturation in the rough endoplasmic reticulum and the different lamellae of the Golgi apparatus were altered in the leukemic cell line, where a decrease in the incorporation of galactose and sialic acid into the AChE structure was mainly observed. AChE glycosylation is essential from the point of view of its activity and circulatory half-life (66). N-glycosylation may play a specific role in the unresolved noncholinergic roles of cholinesterases in cellular differentiation and development (67).

Sialic acid incorporation and its role in the pharmacokinetic properties of proteins was first reported by Morell *et al* (68), who discovered that sialylation alters the circulatory half-life of proteins by protecting the penultimate galactose residues, which are recognized by the asialoglycoprotein receptor (ASGPR). The binding of glycoproteins to ASGPR initiates protein degradation in the liver (69-71). Several studies have been built on the discovery by Morell *et al* (68) by increasing the level of sialic acid in various proteins, including cholinesterases, to prolong their circulatory retention time (62,71,72).

The present study was limited, using only one leukemia cell line (Jurkat). However, it was considered that the results obtained are a valuable starting point for future studies. The authors continue to investigate the possible participation of AChE and other components of the nonneuronal cholinergic system in cell growth and proliferation, allowing the development of new proposals for therapeutic targets against acute lymphoblastic leukemia.

Future research will involve a wider range of leukemia cell lines and clinical samples. Additionally, it will include data on activated normal T lymphocytes to gain a comprehensive understanding of the role of AChE in different functional states of lymphocytes. Notably, future studies of AChE expression in samples from patients with leukemia may allow the use of AChE as a tool for the early detection of leukemia, with the aim on increasing the effectiveness of current treatments.

The results of the present study showed that the content and composition of AChE are altered in Jurkat leukemic cells compared with normal T lymphocytes, from the change in the expression pattern of the transcripts to the decrease in the specific activity of AChE, the differences in glycan processing and the alteration in the assembly of molecular shapes. This opens the way to explore the possible participation of AChE in the proliferation and differentiation of lymphoid cells, as well as in the development of new therapeutic targets against acute lymphoblastic leukemia and the creation of timely detection methods. Further studies should be conducted in other leukemia cell types to elucidate the underlying mechanisms involved in the generation of the proposed molecular targets.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

Data acquisition, processing and validation was performed by LF-L, JG-O, SE-F, GL-V, ID-D, RV-R and IG-T; the study investigation was carried out by LF-L, JG-O and SE-F carried out project administration; LF-L and JG-O wrote the original draft of the manuscript; LF-L, JG-O, SE-F, GL-V, ID-D and IG-T reviewed and edited the manuscript. SE-F, GL-V, ID-D, IG-T, RL-D, RV-R, RV-Q and MM confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The research ethics committee of the National Institute of Pediatrics approved the use of blood samples donated by healthy adults with a registration in the National Bioethics Commission with the number CONBIOETICA-09-CEI-025-20161215 of the National Institute of Pediatrics, Mexico.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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