# Expression and Subcellular Localization of a Novel Nuclear Acetylcholinesterase Protein<sup>\*</sup>

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Acetylcholine is found in the nervous system and also in other cell types (endothelium, lymphocytes, and epithelial and blood cells), which are globally termed the non-neuronal cholinergic system. In this study we investigated the expression and subcellular localization of acetylcholinesterase (AChE) in endothelial cells. Our results show the expression of the 70-kDa AChE in both cytoplasmic and nuclear compartments. We also describe, for the first time, a nuclear and cytoskeleton-bound AChE isoform with  $\sim$ 55 kDa detected in endothelial cells. This novel isoform is decreased in response to vascular endothelial growth factor via the proteosomes pathway, and it is down-regulated in human leukemic T-cells as compared with normal T-cells, suggesting that the decreased expression of the 55-kDa AChE protein may contribute to an angiogenic response and associate with tumorigenesis. Importantly, we show that its nuclear expression is not endothelial cell-specific but also evidenced in non-neuronal and neuronal cells. Concerning neuronal cells, we can distinguish an exclusively nuclear expression in postnatal neurons in contrast to a cytoplasmic and nuclear expression in embryonic neurons, suggesting that the cell compartmentalization of this new AChE isoform is changed during the development of nervous system. Overall, our studies suggest that the 55-kDa AChE may be involved in different biological processes such as neural development, tumor progression, and angiogenesis.

Acetylcholinesterase (AChE; EC 3.1.1.7)<sup>2</sup> is responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh) and therefore the termination of the neural impulse (1). In the last years, it has been shown that the cholinergic system is not confined to the nervous system, but is ubiquitous, leading to the concept of "non-neuronal cholinergic system". AChE, as well as the enzyme which catalyzes the synthesis of ACh, choline acetyltransferase (ChAT), the ACh, or its receptors can all be found in a wide variety of cellular systems such as epithelial cells, blood cells, and vascular endothelial cells (ECs) (2–7). Although its function is not yet clarified, the non-neuronal cholinergic system appears to be involved in the regulation of several biological functions, such as proliferation, differentiation, organization of the cytoskeleton, cell-cell contact, or immune functions (7). Regarding its role in ECs, it was described that nicotine stimulates EC proliferation (8) and induces angiogenesis (9) through nicotinic ACh receptors.

Importantly, AChE expression may be modulated in a tissuespecific manner, and as described, different cellular types express different AChE forms. This may occur because a single AChE pre-mRNA gives rise to three distinct AChE variants generated by 3' alternative splicing (10-12). All of these three variants contain the exons E1, E2, E3, and E4 at its N terminus, diverging in their C terminus sequence (11). The synaptic form (AChE-S), which is the most frequent splicing variant in brain and muscle cells, adds just one more exon to the common core, E6. The other two AChE variants have also E5 in their sequence, although the readthrough (AChE-R) differs from the erythrocyte (AChE-E) by the possession of an additional intron I4 (13). The three alternative transcripts with distinct C termini are translated into three different proteins that differ in their noncatalytic activities, independent of their ACh-hydrolyzing capacity of AChE.

Furthermore, an additional level of diversity is enabled by the existence of novel 5' alternative AChE transcripts (14–16). Recently, it was also described that the 5' splice variations originate N-terminally extended membrane variant(s) of AChE in brain neurons and hematopoietic cells (17). Therefore, the existence of 5' alternative splicing patterns extends the complexity of AChE mRNA transcripts and their protein products.

Several commercially available anti-AChE antibodies yield a doublet around 66–70 kDa (18–20). In ECs, in particular, we have recently demonstrated a predominance of AChE over other cholinesterases and showed the existence of a membrane-bound AChE, which was detected as a doublet of 66–70 kDa using an N-terminal AChE antibody (21). So far, no work

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<sup>&</sup>lt;sup>2</sup> The abbreviations used are: ACh, acetylcholine; AChE, acetylcholinesterase; ChAT, choline acetyltransferase; AChE-S, synaptic acetylcholinesterase; AChE-R, readthrough acetylcholinesterase; AChE-E, erythrocyte acetylcholinesterase; EC, endothelial cell; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cells; HMEC-C, human cardiac microvascular endothelial cells; NGF, nerve growth factor; Pipes, 1,4piperazinediethanesulfonic acid; RT, reverse transcription; WB, Western blot.

has been done regarding AChE cell internalization. In the present report, we were interested in investigating AChE expression and subcellular localization in ECs. Our results reveal the expression of the 70-kDa AChE in both nuclear and cytoplasmic compartments and a new AChE isoform with  $\sim$ 55 kDa that has an exclusively nuclear and cytoskeleton-bound expression. Moreover, we show that an angiogenic stimulus such as vascular endothelial growth factor (VEGF) specifically induces the down-regulation of the 55-kDa AChE isoform in a proteosome dependent manner. In addition, human leukemic T-cells show decreased levels of expression of the novel AChE isoform.

We also verified that this new 55-kDa AChE protein has the same expression pattern in non-neuronal and neuronal cells. Additionally, our results, using cortical neurons from Wistar rats, suggest that the cell compartmentalization of this new 55-kDa AChE isoform is probably changed during the development of the rat brain.

# **EXPERIMENTAL PROCEDURES**

# Cell Culture

Primary human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Shahin Rafii (Cornell University Medical College, NY). Primary human cardiac microvascular endothelial cells (HMEC-C) were purchased from Cambrex. HUVEC and HMEC-C were cultured in complete endothelial medium, as provided by the manufacturer (Cambrex). Upon reaching confluence, the cells were passed onto other gelatin-coated flasks or coverslips (see below) and used up to passage 7 in the experiments described in the present report. VEGF (used at 20 ng/ml) and LLnL (used at 10  $\mu$ M) were purchased from Sigma. The VEGF receptor-2-specific inhibitor (KDRi, used at 70 nM) was obtained from Calbiochem.

Human erythroleukemia (K562), B-cell lymphoma (Daudi), and T-cell leukemia (JURKAT and MOLT-4) cell lines were grown in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum (Invitrogen). The primary leukemialike T-ALL cell line TAIL7 (22) was grown in the presence of 10 ng/ml IL-7. Normal thymocytes or the primary leukemia sample was obtained from thymic samples or from the peripheral blood of a T-ALL patient at diagnosis, respectively.

Stock cultures of PC12 (rat pheochromocytoma) were purchased from ATCC (American Type Culture Collection) and grown in RPMI 1640 medium supplemented with 10% fetal calf serum. For PC12 differentiation-like neurons, the cells were plated 200,000 cells/cm<sup>2</sup>, and the differentiation was induced over ~7 days in RPMI 1640 medium by adding 50 ng/ml nerve growth factor (NGF, Alamone Labs, Israel).

Brain cortical neurons were obtained from 15 to 16-days-old embryos of Wistar rats according to the method described by Agostinho and Oliveira (28). The cells, after being isolated and plated on poly-L-lysine plates, were cultured in neurobasal medium supplemented with 2 mM glutamine, 2% B27 supplement (Invitrogen). The cells were maintained in culture for 7 days before experimental use. In serum-free neurobasal medium supplemented with B27, glial growth is reduced.

Postnatal neurons were obtained from cerebral cortices of 2–5-day-old neonates of Wistar rats, using the same isolation

procedures described to obtain the cortical neurons from embryo. Similarly, the cells were cultured in neurobasal medium supplemented with 2 mM glutamine, 2% B27 supplement (Invitrogen), penicillin (100 units/ml), and streptomycin (100  $\mu$ g/ml), and maintained in culture for 7 days.

Astrocytes were prepared from cerebral cortices of 2–5-dayold neonates of Wistar rats as described previously by Abe and Saito (29). Briefly, cortical hemispheres were trypsinized (0, 25%), dissociated mechanically, and cultured on poly-L-lysine flasks, using DMEM medium (Sigma) supplemented with 10% fetal calf serum. The cells were maintained in culture for 14–15 days, changing the medium every 3–4 days, until confluence was reached. Then the cultures were shaken at 120 rpm for 15 min to remove microglia cells from culture. The medium containing the detached cells was removed, and the purified cultures of astrocytes were maintained 1–2 days before being used. Immunocytochemistry analyses, using specific markers for astrocytes (anti-GFAP) and microglia (anti-Cd11b), showed that these cultures displayed about 98% of astrocytes and 2% of microglia.<sup>3</sup>

# Whole, Cytoplasmic, and Nuclear Protein Extraction, Immunoprecipitation, and Western Blotting

Whole, cytoplasmic, and nuclear protein extracts were prepared as described (23). Nuclear extracts were diluted to have 150 mM NaCl and used to immunoprecipitate. Lysates were pre-cleared with 50  $\mu$ l of protein G-Sepharose beads. Supernatants were incubated with the specific antibody (goat anti-AChE (N-19) antibody) for 12 h, at 4 °C, and incubated with protein G-Sepharose beads for an additional hour at 4 °C. Beads were washed in a lysis buffer and resuspended in SDS loading buffer before electrophoresis. For Western blotting, equal protein amounts were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated with antibodies raised against ChAT (Chemicon) and human (N-19) or rat (E-19) AChE (Santa Cruz Biotechnology). For the competitive assay, N-19 peptide immunogen was supplied by the manufacturer, and the experiment was performed according to the manufacturer's instructions. The recombinant human AChE protein was purchased from Sigma.

# Separation of Whole Cell Protein into Soluble and Insoluble Cytoskeleton Fractions

Cells were resuspended in 100  $\mu$ l of PHEM buffer (60 mm Pipes, 2 mm Hepes, 10 mm EGTA, 2 mm MgCl<sub>2</sub>, and protease/ phosphatase inhibitors) containing 0.1% (v/v) Triton X-100. After 2 min on ice, the Triton X-100 soluble and insoluble fractions were centrifuged for 30 min at 4 °C and 9800 × g. Supernatant (Triton X-100 soluble fraction) was removed to a new tube and diluted 1:1 with 2× reduced sample buffer. The pellet (Triton X-100 insoluble cytoskeleton fraction) was washed with PHEM buffer containing 0.1% Triton X-100 to eliminate the residual soluble element. The cytoskeleton fraction was then resuspended in 100  $\mu$ l of PHEM buffer and diluted 1:1 with 2×



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reduced sample buffer. Insoluble fraction was boiled for 5 min and then centrifuged for 5 min at 4 °C and 9800  $\times$  *g*.

#### Confocal Microscopy

For these experiments, HUVEC were cultured on ethanoltreated, gelatin-coated, glass coverslips, which were placed in 24-well plates. The cells were fixed in 1% (v/v) formaldehyde/ phosphate-buffered saline for 10 min at room temperature and washed in phosphate-buffered saline. After permeabilization with 0.1% (v/v) Triton X-100, the cells were incubated with the primary antibody for 12 h at 4 °C (goat anti-AChE (N-19), Santa Cruz Biotechnology). After primary antibody incubation, the cells were washed and incubated with the secondary antibody (Alexa fluor 488; Molecular Probes) for two hours at room temperature. The samples were mounted in Vectashield and analyzed by confocal microscopy. Sets of optical sections with 0.5- $\mu$ m intervals along the *z* axis were obtained from the bottom to the top of cells using a laser scanning confocal microscope (True Confocal Scanner Leica TCS SP2; Leica Microsystems); objectives HCX PL APOCS  $63 \times 1.4$  oil. The laser lines relative powers were set to have the identical light intensity for the same sample. This was performed using the fieldmaster (Coherent) with the head LM2. Acquisition and image treatment were performed with the LSC software (Leica). The experiments were repeated at least 3 times.

## RT-PCR Assay

Total RNA was obtained using TRIzol<sup>®</sup> (Invitrogen) and treated with DNAfree<sup>TM</sup> (Ambion Inc.) according to the manufacturer's protocol. One  $\mu$ g of total RNA was converted to cDNA by incubation with 200 units of SuperScript II reverse transcriptase (Invitrogen) and 3  $\mu$ g/ $\mu$ l random hexamers in 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 100 mM dithiothreitol, and 0.5 mM dNTPs at 37 °C for 90 min. PCR for AChE was performed using 3  $\mu$ l of cDNA as a template and the following primers: 1522(+), 5'-CGG GTC TAC GCC TAC GTC TTT GAA CAC CGT GCT TC-3'; 2003(–), 5'-CAC AGG TCT GAG CAG CGA TCC TGC TTG CTG-3'; 1917(–), 5'-ATG GGT GAA GCC TGG GCA GGT G-3'.

To detect the mRNA of the synaptic AChE including exons E2-E3-E4-E6, we employed the 1522 (+)/2003(-) primer pair, and the PCR was provided at 35 cycles (45 s at 95 °C, 45 s at 56 °C, and 1 min at 72 °C). The expected PCR product was 452 bp.

PCR for AChE using the 1522 (+) and 1917 (-) primer pair was performed at 35 cycles (45 s at 95 °C, 45 s at 60 °C, and 1 min at 72 °C). Two PCR products, 387 bp (AChE-E) and 469 bp (AChE-R), could be generated. Primer 1 (5'-CAATGAC-CCCTTCATTGACC) and primer 2 (5'-TGGGATTTCCATT-GATGACA) were used to generate a 122-bp fragment of glyceraldehyde-3-phosphate dehydrogenase as internal control.

## Peptide Mass Fingerprinting Determination

*In-gel Digestion*—Excised gel bands from SDS-PAGE were washed with 50% acetonitrile and dried by centrifugation under vacuum. Digestion was performed as described by Spenglen (24). For the reduction and carbamidomethylation of cysteines, gel pieces containing the proteins were treated with dithiothreitol and iodoacetamide. Modified trypsin was added to the

# A Novel Nuclear Acetylcholinesterase Protein

dried gel pieces and incubated at 37 °C overnight. Desalting and concentration of supernatant from trypsin digestion was carried out (after acidification with formic acid) with custom-made chromatographic microcolumns containing POROS 10 R2 material filling (20- $\mu$ m bead size) equilibrated with 2% trifluoroacetic acid (25). Peptides were directly eluted to the MALDI plate using  $\alpha$ -CHCA in 70% acetonitrile with 0.01% trifluoroacetic acid.

*MALDI-TOF Mass Spectrometry*—All mass spectra were obtained using a PerSpetive Biosystems MALDI-TOF mass spectrometry Voyager-DE<sup>TM</sup> STR equipped with delayed extraction, reflectron, and with a 337-nm nitrogen laser. External mass calibration was performed with a mixture of peptide standards PepMix1. Monoisotopic peptide masses were used to compare peptide map profiles.

# RESULTS

*Expression and Subcellular Localization of AChE in HUVEC and HMEC-C*—With the aim of studying the expression and cell localization of AChE protein in ECs, cytoplasmic and nuclear extracts (Fig. 1*A*, *left panel*) and soluble and insoluble cytoskeleton fractions (Fig. 1*A*, *right panel*) were prepared from HUVEC and HMEC-C. AChE immunoblottings were performed using antibodies raised against the N terminus (N-19) or C terminus (H-134) of human AChE. Although both AChE antibodies recognize the 70-kDa AChE present in all of the subcellular protein fractions, only the N terminus antibody (N-19) can detect a protein with ~55 kDa in the nuclear and insoluble cytoskeleton fractions (Fig. 1*A*). A human AChE recombinant protein was used as a control, and a protein of 70 kDa (AChE) was detected with both AChE antibodies.

The same membranes were incubated with lamin B (nuclear protein) or vimentin (cytoskeleton protein) antibodies, demonstrating the lack of contamination in the cytoplasmic and soluble fractions, respectively. The nuclear AChE localization in HUVEC was confirmed by confocal microscopy (Fig. 1*B*).

AChE mRNA and Protein Expression in Neuronal and Nonneuronal Cells—To investigate whether the nuclear and cytoskeleton-bound 55-kDa protein, recognized by the antibody N-19, is endothelial cell-specific, we used several human cell lines and rat brain primary cell cultures (cortical neurons and astrocytes). The expression of AChE transcript by RT-PCR was confirmed in all cell types, using selective primers for each of the alternatively spliced forms of AChE mRNA (11).

In mammalian cells, all AChE transcripts contain exon 1 (E1), exon 2 (E2), exon 3 (E3), exon 4 (E4), and exon 6 (E6), but exon 5 (E5) is variably spliced. The synaptic form does not contain E5, differing from the erythrocytic and readthrough forms. These two last variants only diverge between them by the presence of intron 4 (I4) in the readthrough form (13).

As shown in Fig. 2*A*, using the 1522 (E4) and 2003 (E6) pair primer, a fragment of 452 bp (corresponding to the synaptic isoform) was detected in Daudi, Jurkat, K562 (*lanes 3–5*), and in non-differentiated and NGF-differentiated PC12 (*lanes 7* and 8) human cell lines, as well as in postnatal rat astrocytes (*lane 6*) and cortical neurons (*lane 9*). Simultaneously, we used the HUVEC (Fig. 2*A*, *lane 2*), the vector pGEM-AChE-E6 as a positive control (*lane 10*) and nuclease-free water as a negative



FIGURE 1. **AChE expression in ECs.** *A*, cytoplasmic (*C*), nuclear (*N*), insoluble (*I*), and soluble (*S*) extracts were prepared from HUVEC and HMEC-C and analyzed by WB with an antibody against the N terminus (*N*-19) or C terminus (*H*-134) of human AChE or with anti-lamin B and anti-vimentin antibodies. Note the presence of a protein with  $\sim$ 55 kDa in the nuclear and insoluble fractions using the N-19 AChE antibody. *B*, confocal microscopy of HUVEC cultured on gelatin-coated slides was performed using the N-19 antibody. Internal section (*S*/10) demonstrates a nuclear localization of AChE. Magnification, ×630.



FIGURE 2. **Expression of alternatively spliced transcripts of AChE.** AChE gene expression in HUVEC, Daudi, Jurkat, K562, rat astrocytes, non-differentiated (*ND*), and NGF-differentiated (*D*) PC12, and rat neurons. *A*, synaptic AChE transcript (452 bp) was detected in all cell lines and in primary cell cultures (*lanes 2–9*). pGEM-AChE-E6 vector (*lane 10*) and nuclease-free water (*lane 11*) were used as positive and negative controls, respectively, in RT-PCR reactions. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a mRNA quantity control in non-differentiated and differentiated PC12 cell line. *B*, erythrocytic AChE transcript (387 bp) was detected in K562, Jurkat, and HUVEC (*lanes 3–5*), whereas readthrough AChE transcript (469 bp) was only detected in Jurkat cell line (*lane 4*). No AChE transcript was detected in Daudi cell line (*lane 2*). pGEM-AChE-I4/E5 vector (*lane 6*) and nuclease-free water (*lane 7*) were used as positive and negative controls, respectively, in RT-PCR reactions.

control (*lane 11*). An increase in the amount of 452-bp AChE transcript after PC12 differentiation in neurons was observed. Thus, our results confirm the expression of the synaptic AChE mRNA in all cellular types studied.

In addition, we investigated the presence of the readthrough and erythrocytic AChE variants only in the human cell lines because E5 is not present in rat mRNA AChE sequences. As shown in Fig. 2*B*, a 387-bp fragment (corresponding to the erythrocyte isoform) was detected in K562, Jurkat, and HUVEC cells (*lanes* 3–5) by PCR using the 1522 (E4) and 1917 (E5) pair primers. Additionally, a fragment of 469 bp (corresponding to the readthrough isoform) was also detected in Jurkat cells (*lane* 4), whereas in Daudi cells none of the transcripts were detected (*lane* 2). The vector pGEM-AChE-I4/E5, a positive control of the readthrough isoform (Fig. 2*B*, *lane 6*), and a negative RT-PCR control (*lane 7*) were also used. The results suggest the expression of the erythrocyte AChE variant in HUVEC and K562 cells and the expression of both erythrocyte and readthrough AChE variants in Jurkat cells.

Knowing that all cell types studied by us expressed at least one AChE splice variant, we examined the expression of AChE protein. As shown in Fig. 3A by Western blot using the N-19 AChE antibody, the protein of 70 kDa was detected in the nucleus, cytoplasm (upper panel), and soluble and insoluble cytoskeleton fractions (*lower panel*) of K562, Jurkat, and Daudi cells. As already seen in ECs, an exclusively nuclear and insoluble protein of 55 kDa is also detected in all of the human cell lines. To know whether this 55-kDa isoform is also detected in the cells of the nervous system and PC12 cells, we used a specific antibody against the N terminus of rat AChE (E-19 antibody), which is described to recognize two AChE proteins with 82 and 69 kDa. We observed that postnatal neurons and astrocytes, as well as non-differentiated and NGF-differentiated PC12 cells expressed both 82- and 69-kDa AChE proteins (Fig. 3B, upper and lower panels). Interestingly, using the antibody against the N terminus of the rat AChE, the protein of 55 kDa is detected once more only in the nuclear (Fig. 3B, upper panel) and insoluble fractions (lower panel). All of the membranes were incubated with an antibody

against lamin or vimentin to confer protein extraction specificity. Because Daudi cells did not express vimentin, insoluble and soluble Daudi extracts were incubated with an anti-clathrin antibody.

The Expression of a Nuclear AChE Protein with ~55 kDa— An additional protein of ~55 kDa was detected using an antibody against the N terminus of human or rat AChE sequence. To confirm the specificity of the N-19 antibody, a competitive assay was performed. In this assay, the N-19 AChE antibody was pre-incubated with different doses of N-19 peptide (commercially available). As shown in Fig. 4*A*, increasing pre-adsorptions of N-19 with its peptide antigen blocked the reactivity of the 70-kDa AChE and of the 55-kDa protein. Moreover, an AChE immunoprecipitation assay was also carried out. With this



FIGURE 3. AChE protein expression in neuronal and non-neuronal cells. Cytoplasmic (C) and nuclear (N) extracts (upper panels) and insoluble (I) and soluble (S) fractions (*lower panels*) were prepared. A, WB analysis with N-19 anti-AChE antibody or with anti-lamin B, anti-vimentin, and anti-clathrin antibodies in human cell lines. B, WB analysis using the E-19 anti-AChE antibody or anti-lamin B and anti-vimentin antibodies in rat cells. Note the presence of an ~55-kDa protein using the N-19 or E-19 anti-AChE antibody in the nuclear and insoluble fractions.

purpose, nuclear extracts of HUVEC were prepared, and 500  $\mu$ g of protein was used to perform AChE immunoprecipitation followed by Western blot. Both immunoprecipitation and immunoblotting were conducted with the N-19 AChE antibody (Fig. 4*B*). A nuclear control lysate (Fig. 4*B*, *lane 3*) and a negative immunoprecipitation control (*lane 1*) were also included in the experiment. The results obtained confirm the precipitation of both 70- and 55-kDa proteins in a nuclear extract.

For protein identification by mass spectrometry a new AChE immunoprecipitation was carried out. The immunoprecipitates were applied to SDS-PAGE, and the resolved protein bands were visualized with mass spectrometry-compatible silver staining. Both 70- and 55-kDa protein bands were removed and tryptic digested. Mass spectra of the concentrated and desalted tryptic peptides mixtures were collected by MALDI-TOF mass spectrometer. Six and twelve m/z peaks with relevant intensities were observed in the 55- and 70-kDa digests, respectively. Five of these peaks are common to both mass spectra (see Fig. 4*C*).

The 55-kDa AChE Protein Is Down-regulated in Response to VEGF—To investigate the role of the 55-kDa AChE protein in ECs, we decided to investigate the modulation of its expression in response to an angiogenic stimulus. Therefore, the HUVEC

# A Novel Nuclear Acetylcholinesterase Protein



FIGURE 4. The expression of a nuclear AChE protein with ~55 kDa. A, nuclear extracts from HUVEC were prepared, and equivalent amounts of nuclear protein, 50 µg/lane, were run on SDS-polyacrylamide gel. *Lane 1* was incubated with the N-19 antibody, whereas *lanes 2* and 3 were incubated with an N-19 antibody/N-19 peptide mixture. *Lane 2* differs from *lane 3* in the amount of N-19 peptide used in the mixture, as indicated in the figure. Note that both 70- and 55-kDa proteins failed to be detected with progressive higher pre-adsorptions of N-19 with its peptide antigen. *B*, immunoprecipitation (*IP*) of HUVEC nuclear extract (*N*) was conducted with the N-19 anti-AChE antibody followed by N-19 AChE immunoblotting (*lane 2*). Immunoprecipitation negative control (*IgG*) (*lane 1*) and a nuclear extract (*lane 3*) were also included in the experiment. Note that both 70- and 55-kDa proteins were immunoprecipitated. *C*, mass spectra of 70-kDa AChE form tryptic digest. The *m/z* peaks with equivalent values in the mass spectra of 55-kDa AChE form tryptic digest are marked with an *asterisk*.



FIGURE 5. **The 55-kDa AChE protein expression in response to VEGF stimulus.** HUVEC were cultured in the absence of serum and growth factors during 24 h and then not incubated (*lanes 1* and 2) or incubated with a VEGF receptor-2 inhibitor (*lane 3*) for 12 h or a proteosome inhibitor (*lane 4*) for 15 min. Finally, the ECs were stimulated (*lanes 2–4*) or not (*lane 1*) with VEGF for 30 min, and whole protein extracts were prepared and analyzed by WB using the N-19 anti-AChE antibody.

were cultured in the absence of serum and growth factors during 24 h and then incubated or not with a VEGF receptor-2 inhibitor (KDRi) for 12 h or a proteosome inhibitor (LLnL) for 15 min. Finally, the cells were stimulated with VEGF for 30 min, and whole protein extracts were analyzed by WB (Fig. 5). Our results suggest that the level of the 55-kDa AChE protein is decreased after VEGF stimulation (Fig. 5, *lane 2*), a mechanism that is inhibited by a specific VEGF-receptor-2 inhibitor (*lane 3*). Moreover, VEGF effect in the level of the 55-kDa AChE expression is inhibited in HUVEC pre-treated with the proteosome inhibitor LLnL (Fig. 5, *lane 4*). Interestingly, our results show that the 70-kDa AChE protein is not regulated by VEGF.

*The 55-kDa AChE Protein Expression in Human T-cells*—We next investigated whether expression of 55-kDa AChE could be

altered in tumor cells. We compared human T-ALL primary cells and cell lines with their normal counterparts. Whole protein extracts were prepared from these cells, and AChE expression was analyzed by WB (Fig. 6). As shown the level of the 55-kDa AChE expression is decreased in the leukemic T cell lines (Fig. 6, *lanes* 5-7) and in the primary T-ALL sample (*lane* 4) when compared with normal primary thymocytes (*lanes* 1-3). In contrast, the level of the 70-kDa AChE is similar in both leukemic and normal cells.

AChE Cell Compartmentalization during Rat Brain Development—To investigate whether the nuclear expression of the 55-kDa AChE protein is always present during rat brain development, cortical neurons from embryo (15–16 days) of and neonatal (2-5 days) Wistar rats were obtained and maintained in culture for 7 days. Then, cytoplasmic and nuclear extracts were prepared from both neuron cultures and analyzed by Western blot using the E-19 AChE antibody. As can be seen in Fig. 7*A*, the 55-kDa AChE protein is detected both in cytoplasm and nuclear extracts in embryonic neurons, in contrast to an exclusively 55 kDa nuclear AChE expression found in postnatal neurons. Using the same neuronal cultures from embryonic or postnatal rats, soluble and insoluble cytoskeleton extracts were prepared. By E-19 immunoblotting (Fig. 7B), we can detect the presence of the 55-kDa AChE protein in soluble and cytoskeleton extracts from embryonic neurons. As expected, in neurons from postnatal rats, the 55-kDa AChE protein is only evident in insoluble extracts. All the extracts were analyzed with lamin B (nuclear protein) or vimentin (insoluble protein) with the objective of guaranteeing protein extraction specificity.

# DISCUSSION

The present study reveals novel aspects about the expression and subcellular localization of AChE protein, an important



FIGURE 6. **The 55-kDa AChE protein expression in normal and leukemic T-cells.** Whole protein extracts were prepared from normal thymocytes (*lanes 1–3*), a primary T-cell acute leukemic sample (*lane 4*), and leukemic human T-cell lines (*lanes 5–7*). The expression of AChE protein was investigated using the N-19 anti-AChE antibody.



FIGURE 7. AChE protein expression in prenatal and postnatal Wistar rats. A, cytoplasmic (C) and nuclear (N) extracts were prepared and analyzed by WB using E-19 anti-AChE (upper panels) and anti-lamin B (lower panels) antibodies. B, insoluble (I) and soluble (S) fractions were prepared and analyzed by WB with the E-19 anti-AChE (upper panels) and anti-vimentin (lower panels) antibodies.

component of the cholinergic system. In the present report we observe a cytoplasmic, nuclear, and cytoskeleton expression of the classical AChE proteins with  $\sim$ 70 kDa in both HUVEC and HMEC-C.

AChE has been described to be expressed in the cytoplasm at the initiation of apoptosis and then in the nucleus of apoptotic bodies upon commitment to cell death (26). The apoptosis was induced by various stimuli in different cell lines, including swine and bovine ECs (26). In contrast with these published results, our data clearly show a nuclear 70-kDa AChE expression in non-apoptotic human culture ECs, using an antibody against the C- or N-terminal of AChE.

Concerning the nuclear expression of the 70-kDa AChE proteins with distinct C termini, we also investigated the prediction of their subcellular localization using the bioinformatic program PSORT II; and for any of the three AChE isoforms the result was about 13–17%. According to our experimental results, we were expecting to find higher values; however, the prediction was done using protein sequences according to 3' splicing variants, and neither the sequence diversity in 5' alternative AChE transcripts (17) nor the post-translational modifications were considered.

Furthermore, we also describe a new AChE protein with  $\sim$ 55 kDa that was only detected using a commercial antibody raised against the N-terminal portion of AChE. By different technical approaches, such as competitive assays and AChE immunoprecipitation, our presented data indicate that the 55-kDa protein is an AChE-specific protein, possibly truncated in its C-terminal region. The peptide maps obtained by mass spectrometry for both protein forms (70 and 55 kDa) also support this hypothesis. The 55-kDa AChE protein may be originated by post-translation modification; however, alternative promoter usage combined with alternative splicing extends the diversity and complexity of AChE mRNA transcripts and their protein products (17).

We demonstrate that this 55-kDa AChE protein is expressed in ECs, and its level of expression is modulated by an angiogenic stimulus. Therefore, in response to VEGF a decrease in the expression of the 55-kDa AChE is observed, and this process is mediated by VEGF receptor-2 signaling through the proteosome pathway. Our findings show a relationship between the down-regulation of this new 55-kDa AChE isoform and an angiogenic response, whereas the 70-kDa AChE protein seems not to be involved. Although as far as we know there are no reports on AChE involvement in angiogenesis, there is

evidence that signaling via the nicotinic ACh receptors promotes angiogenesis (9).

Here we show that the 55-kDa AChE isoform, a new component of the non-neuronal cholinergic system, may have a significant relevance in angiogenesis. These findings raise the possibility that decreased expression of the novel AChE form may contribute to tumor development by affecting tumor cells directly or by promoting



angiogenesis. Interestingly, in human leukemia T-cells, the 55-kDa AChE expression is down-regulated when compared with normal T-cells.

Moreover, we show the 55-kDa AChE protein is expressed not only in ECs but also in other cells from the neural and non-neuronal systems. Moreover, all cell types in this study present nuclear and insoluble cytoskeleton localizations for the 55-kDa AChE protein, whereas the 70-kDa AChE has a nucleocytoplasmic expression. Concerning neuronal cells we can distinguish an exclusively nuclear localization of the 55-kDa protein in postnatal neurons in contrast to prenatal neurons in which equal amounts of 55-kDa AChE isoforms are detected both in the cytoplasm and in the nucleus. The results suggest that during development, this new 55-kDa AChE protein presents a subcellular compartmentalization. Future studies should address to the existence of distinct temporal changes in the subcellular localization of this protein.

For cholinergic neurons in human brain, it is described (27) that the 69-kDa ChAT protein is localized in both nuclear and cytoplasmic compartments, whereas the 82-kDa isoform is found predominantly in neuronal nuclei. However, the subcellular localization of the 82-kDa ChAT changes to assume a subcellular pattern that resembles that of the 69-kDa ChAT with increasing age and in Alzheimer disease (27). It will be very interesting to analyze the pattern of expression or subcellular distribution of AChE protein, mainly for the 55-kDa compartmentalized isoform in neurodegenerative diseases.

In summary, this study shows novel information about the expression and cell localization of AChE in ECs, revealing at the same time a new nuclear and cytoskeleton-bound AChE isoform, with  $\sim$ 55 kDa, which seems to be expressed not only in ECs but also in the neuronal and the non-neuronal system. In response to an angiogenic stimulus such as VEGF, a decrease of the 55-kDa AChE protein expression is observed, and a clear relationship between this expression and an angiogenic response is evident. Because we demonstrated the biological relevance of this new AChE protein isoform, ongoing studies are focusing on the mechanisms whereby the 55-kDa AChE protein plays a role in angiogenesis. This work brings a new perspective to the functional role of these different compartmentalized isoforms, suggesting that their cell localization and expression may be probably changed in a specific moment during the development of nervous system.

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