Blood Cells, Molecules and Diseases 59 (2016) 77–84

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

Cholinergic activation enhances retinoic acid-induced differentiation in the human NB-4 acute promyelocytic leukemia cell line

Sadudee Chotirat [a], Tawit Suriyo [b], Marianne Hokland [c], Peter Hokland [d], Jutamaad Satayavivad [b,e], Chirayu U. Auewarakul [f,*]

[a] Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
[b] Laboratory of Pharmacology, Chulabhorn Research Institute, Bangkok 10210, Thailand
[c] Department of Biomedicine, Faculty of Health, Aarhus University, Aarhus 8000, Denmark
[d] Department of Haematology, Aarhus University Hospital, Aarhus 8000, Denmark
[e] Center of Excellence on Environmental Health and Toxicology, Office of Higher Education Commission, Ministry of Education, Bangkok 10400, Thailand
[f] Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

ARTICLE INFO

Article history:
Submitted 22 December 2015
Revised 16 April 2016
Accepted 17 April 2016
Available online 20 April 2016

Editor: Mohandas Narla

Keywords:
Non-neuronal cholinergic system
Carbachol
NB-4 cells
CD11b
Differentiation

ABSTRACT

The non-neuronal cholinergic system (NNCS) has been shown to play a role in regulating hematopoietic differentiation. We determined the expression of cholinergic components in leukemic cell lines by Western blotting and in normal leukocyte subsets by flow cytometry and found a heterogeneous expression of choline acetyltransferase (ChAT), acetylcholinesterase (AChE), choline transporter (CHT), M3 muscarinic acetylcholine receptor (M3-mAChR) and α7 nicotinic acetylcholine receptor (α7-nAChR). We then evaluated NNCS role in differentiation of human NB-4 acute promyelocytic leukemia cell line and discovered a dramatic induction of M3-mAChR after all-trans retinoic acid (ATRA) treatment ($p < 0.0001$). Adding carbachol which is a cholinergic agonist to the ATRA treatment resulted in an increase of a granulocytic differentiation marker (CD11b) as compared with ATRA treatment alone ($p < 0.05$), indicating that cholinergic activation enhanced ATRA in inducing NB-4 maturation. The combination of carbachol and ATRA treatment for 72 h also resulted in decreased viability and increased cleaved caspase-3 expression when compared with ATRA treatment alone ($p < 0.05$). However, this combination did not cause poly (ADP-ribose) polymerase (PARP) cleavage. Overall, we have shown that NB-4 cells expressed M3-mAChR in a differentiation-dependent manner and cholinergic stimulation induced maturation and death of ATRA-induced differentiated NB-4 cells.

1. Introduction

The treatment of leukemia in the developing countries and in countries with restrained economics is a serious stumbling block for traditional cytoreductive measures [1]. Consequently, less expensive modalities are highly sought for. The non-neuronal cholinergic system (NNCS) functions through mediator acetylcholine (ACh) and its components including choline transporter (CHT), muscarinic (mAChRs) and nicotinic receptors (nAChRs: ACh receptors), choline acetyltransferase (ChAT: synthesizing ACh enzyme), and acetylcholinesterase (AChE: degrading ACh enzyme) [2,3]. Importantly, the physiological significance of the NNCS has been documented with respect to growth regulation, differentiation, secretion, barrier functions, immunomodulation, and apoptosis in both healthy and malignant cells including neuroblastoma, lung, colon, cervical, breast, prostate, and bile duct cancers [4–13]. Previously, mAChRs stimulation has also been shown to inhibit K562 chronic myelogenous leukemia cell line proliferation [14,15], while α7-nAChR stimulation has been reported to support HL-60 acute promyelocytic leukemia cell line differentiation [16]. Taken together, these data suggest to us that the cholinergic system could potentially be a modulator of leukemogenesis.

In search of a model system to test this, acute promyelocytic leukemia (APL) could be of value. It represents a distinct subtype of acute myeloid leukemia (AML) [17], in which the balanced reciprocal translocation of chromosome 15 and 17 results in a fusion transcript between the promyelocytic leukemia gene (PML) and retinoic acid receptor-α (RARα) gene [18]. The fusion protein causes a differentiation arrest at the promyelocytic stage in addition to deregulation of apoptotic signaling cascade [19]. Importantly, this differentiation arrest can be lifted by a number of reagents. Thus, the combination of all-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3) targeting aberrant fusion genes has been shown to induce complete remission in up to 95% of APL patients [20,21]. This treatment modality has proven to

* Corresponding author at: Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Wanglang Road, Bangkoknoi, Bangkok 10700, Thailand.
E-mail addresses: sadudee.chotirat@gmail.com (S. Chotirat), tawit@cri.or.th (T. Suriyo), mhokland@biomed.au.dk (M. Hokland), phokland@clin.au.dk (P. Hokland), jutamaad@cri.or.th (J. Satayavivad), chirayuaue@yahoo.com, chirayu.aue@mahidol.ac.th (C.U. Auewarakul).

http://dx.doi.org/10.1016/j.bcmd.2016.04.009
1079-9796/

be a cornerstone for APL therapy in the western countries, where subsequent standard cytoreduction is subsequently applied [22]. In some developing countries, such therapy constitutes the only modality in situations where other subsets of AML cannot be addressed due to economic constraints [1,23,24].

Little has been reported on the expression pattern of cholinergic components during promyelocytic maturation and their modulating regulatory effect on promyelocytic cell functions and cell growth. Consequently, in the present study, we analyzed the expression of cholinergic components in selected leukemic cell lines and human leucocyte subsets, and in particular, explored the functional role of NNCS in the APL-NB-4 cell line comprising cell viability, terminal differentiation, and apoptosis by using the combination of ATRA and carbachol treatment. The data raised should be extended to primary APL cells in order to further evaluate the clinical value of the cholinergic system in leukemogenesis.

2. Material and methods

2.1. Reagents

Carbamylcholine chloride (carbachol), atropine, mecamylamine, all-trans retinoic acid (ATRA), arsenic trioxide (As_2O_3), absolute ethanol (EtOH), and dimetylsulphoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solution of carbachol was prepared in distilled water at 10^3 mM whereas 10^2 mM and 10^1 mM of ATRA and As_2O_3, respectively were prepared in ethanol and stored protected from light at −80 °C. Fetal bovine serum (FBS) was purchased from JR Scientific (Woodland, CA).

2.2. Cell lines

Leukemic cell lines (acute promyelocytic leukemia NB-4 (CLS-300299); chronic myeloid leukemia K-562 (CLS-820700) were purchased from CLS cell line service (Germany); T-lymphocytic leukemia MOLT3 (ATCC® CRL-1552™); B lymphocyte RPMI1788 (ATCC® CCL-156™) were obtained from ATCC®. All cell lines were cultured in RPMI-1640 (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% FBS, 2 mM of L-Glutamine, 1 mM sodium pyruvate, 20 mM D-Glucose, and 100 IU/mL of penicillin and 100 μg/mL streptomycin, (Invitrogen, Carlsbad, CA). SH-SY5Y cell line (ATCC®, CRL-2266™) was cultured in a 1:1 mixture of Ham's F12 and MEM (Gibco, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-Glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. The cells used in the experiments were started at passage 5 and did not exceed passage 30.

2.3. NB-4 cell line differentiation

Briefly, 10 μL of NB-4 cell suspension were well-mixed with Muse™ Count & Viability Cell Dispersal Reagent in 1:20 dilution, and analyzed for the total number of living cells by using the Muse® cell analyzer (Merck Millipore, Darmstadt, Germany). Stock solutions of ATRA and As_2O_3 were further diluted in culture medium to the final concentration (1 μM). The NB-4 cells were induced to granulocytic differentiation by ATRA treatment for 5 days. ATRA-induced differentiated NB-4 cells were counted and maintained at a concentration of approximately 2–5×10^5 cells/mL and half of the medium was replaced and fresh ATRA added every 2 days.

2.4. Cell viability

Cell viability was measured by the XTT cell viability assay according to manufacturer's instruction (Sigma-Aldrich). Briefly, non-differentiated or ATRA-induced differentiated NB-4 cells were generated in 96 well plates (1×10^4 cells/100 μL/well). Cultures were treated with different concentrations of carbachol (0.01–1000 μM) in the medium containing either growth stimulation (supplemented with 10% serum) or deprivation condition (supplemented with 1% serum after cells were deprived of serum for 24 h). To confirm apoptosis of ATRA-induced differentiated NB-4 cells, cultures with ATRA (1 μM) alone were used as controls whereas As_2O_3 (1 μM) was utilized as a positive control for cell death. Twenty, 44, and 68 h post treatment, the cells were added 50 μL of XTT sodium salt ($C_{22}H_{16}N_7NaO_{13}S_2$) diluent mixed with activator phenazinemethosulfate; PMS, $C_{13}H_{11}N_2 \cdot CH_3SO_4$ (Sigma-Aldrich) and subsequently, incubated for another 4 h in 5% CO_2 humidified incubator at 37 °C to achieve 24, 48, and 72 h treatment regimen, respectively. Finally, the optical density (OD) was measured at a wavelength between 450 and 500 nm using a SpectraMax M3 Microplate Reader (Molecular Devices, Sunnyvale, CA) to calculate the percentage of cell viability.

2.5. Flow cytometry

The expression of CD11b, a differentiation marker for granulocytes, was measured by flow cytometry. One million NB-4 cells were harvested, washed twice with cold PBS (pH = 7.6) and incubated with anti-CD11b-PE antibody (clone D12, BD Biosciences, San Jose, CA) for 30 min in the dark at room temperature. Thereafter, the cells were washed twice with cold PBS, resuspended in 500 μL of PBS and analyzed on a FACS Canto flow cytometer (Becton Dickinson, USA) acquiring 3×10^4 events per sample. For AChE expression on primary leukocyte subsets, a total of 5×10^5 mononuclear cells (MNCs) previously blocked with human Ig (CSL Behring AG, Bern, Switzerland) for 30 min in the dark at 4 °C or 100 μL of whole blood (WB) were stained with primary polyclonal rabbit-*anti*-human AChE antibody (Abgent, San Diego, CA) for 15 min and washed twice. The MNC samples were then incubated for another 30 min with goat-*anti*-rabbit PE secondary antibody together with *anti*-CD3 V450 (clone UCHT1, BD Biosciences, San Jose, CA), *anti*-CD19 FITC (clone HD37, Dako, Glostrup, Denmark), *anti*-CD56 (clone CMSSB, eBioscience, San Diego, CA), and Live/Dead dye (Life Technologies, Oregon, CA), washed twice, and fixed with 0.9% formaldehyde. Following *anti*-AChE, the WB samples were further incubated for another 30 min with *anti*-CD11b (clone Bear1, Beckman Coulter, Fullerton, CA) and anti-CD14 (clone MϕP9, BD Biosciences) followed by incubation in FACS Lysis (BD Biosciences) for 10 min, washed once and resuspended in PBS. All samples were analyzed on an LSR Fortessa (Becton Dickinson) acquiring at least $5x10^4$ events per sample. Using single stained compensation beads (eBioscience) a compensation matrix was created. The median fluorescence intensity (MFI) was calculated using the FlowJo software v.10.6 (TreeStar Inc., Ashland, OR).

2.6. Western blot analysis

Approximately 5×10^6 cells were washed twice with cold PBS; pH 7.4 (2.7 mM potassium chloride, 1.8 mM potassium phosphate, 137 mM sodium chloride, 10.1 mM sodium phosphate). The cells were harvested on ice and incubated in 120 μL lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP40, 10 μL/mL PI cocktail, 0.1 mM PMSF, 1 mM Na_3VO_4, 20 mM NaF and 1× protease inhibitor cocktail set I (Calbiochem, Germany). Cell lysates were sonicated and incubated at 4 °C for 30 min prior to centrifugation at 16,000 ×*g*, at 4 °C for 15 min. The supernatant was collected and the protein concentration measured by Bradford reagent (Bio-Rad, Hercules, CA). Aliquot (35 μg) was electrophoresed onto with 7.5% or 12.5% SDS-polyacrylamide gel followed by electrotransfer to nitrocellulose membrane. The nitrocellulose membrane was incubated in blocking buffer for 1 h (5% non-fat dry milk in TBST; 50 mM of Tris, 150 mM of NaCl, 0.1% of Tween-20, pH 7.6). The membrane was incubated with primary antibodies against *anti*-cleave caspase-3; Clone Asp175 (1:1000), *anti*-caspase-3, and *anti*-MMP-9; Clone G657 (1:1000) purchased from Cell Signaling (Beverly, MA). *Anti*-PARP;