Non-neuronal Cholinergic System in Megakaryocytıc Differentiated K562 Cells

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Introduction

Acetylcholine is a neurotransmitter in the nervous system but it serves also as a paracrine or autocrine factor in different cell types, where it is linked to functions like proliferation and cell differentiation. Muscarinic receptors are relatively abundant in the central nervous system and peripheral parasympathetic nervous system (1). Many cells express a mixture of muscarinic receptor transcripts. Changes in muscarinic M_2 and M_3 receptor mRNA levels in response to agonist treatment have been reported in cerebellar granule cells, Chinese hamster ovary cells, lymphocytes and in the human neuroblastoma cell line SH-SY5Y (2,3). Several researchers have suggested that nonneuronal acetylcholine and cholinergic agonists alter cell growth and proliferation of lymphocytes. Costa et al. demonstrated that acetylcholine released from T-lymphocytes acts via the M_3 acetylcholine muscarinic receptor (mAChR) to trigger nuclear signaling and up-regulation of gene expression in T and B-lymphocytes (1). We have previously demonstrated the presence of M_2, M_3 and M_4 mAChRs and M_3 subtype mediated NO signaling in K562 chronic myelogenous leukemic cells (4). We also showed that carbachol (CCh), cholinergic agonist, treatment leads to changes in muscarinic M_2, M_3 and M_4 receptor transcripts as well as M_2 and M_3 protein levels and enhances cyclic adenosine monophosphate (cAMP) accumulation in K562 cells (5,6).

In this study, we investigated the levels of muscarinic receptor protein expression in megakaryocytic differentiated K562 leukemia cells. And we also investigated the effects of agonist stimulation on megakaryocytic differentiated K562 leukemia cells proliferation.

Muscarinic cholinergic receptors activate stimulatory growth mechanisms in megakaryocytic differentiated K562 leukemia cells. And we also investigated the effects of agonist stimulation on cell proliferation and muscarinic receptor expression in megacaryocytic differentiated K562 leukemia cells. We found that CCh changed proliferation of megakaryocytıc dıfferentiated K562 cells proliferation in 24 and 48 hours, These results suggest that CCh may be modulates megakaryocytıc dıfferentiated K562 leukemic cells proliferation through muscarinic acetylcholine receptors.

Materials and Methods

Cell Proliferation Assay. Megakaryocytic differentiation was induced by applying phorbol 12-myristate 13-acetate (PMA) (Calbiochem) at the concentration of 10 nM . Cell proliferation was assessed by measuring BrdU incorporation during DNA synthesis in proliferating cells. 100 ul of passaged Megakaryocytic differentiated K562 cells (1 × 10 4 cells) were seeded into 96 well plates containing RPMI-1640 medium with 0% FBS.After 24 h these “starved cells” were placed into medium containing 1% serum . One of the following was then added: atropine (10 uM), and 30 min later CCh (100u M) was added and left for 24 h and 48h.

Western blot analysis. Anti-Muscarinic antibodies were purchased from Santa Cruz, Inc and M_4 (life span). PMA-induced megakaryocytic differentiation of K562 cells was detected by the expression of integrin beta 3 (Santa Cruz Inc.). For immunoblot assay, 100 microgram of protein from each sample was resolved on a 12% SDS-PAGE gel. Proteins were electrotransferred to nitrocellulose membranes which were exposed to primary antibodies and then to alkaline phosphatase-conjugated secondary antibodies. The antibody–antigen complex was detected with NBT/BCIP. The densitometric analyses were carried out with Bio-Rad Molecular Analyst software (free edition, www.totallab.com).

Statistical analysis. Data are mean ± SEM of N experiments. One way-Anova dunnett’s multiple comprasion test were performed. Statistical tests were performed with the Prism program (Graphpad Software) and $P < 0.05$ was considered significant.

Results

Megakaryocytic Differentiation was observed by inverted microscope (Figure 1).

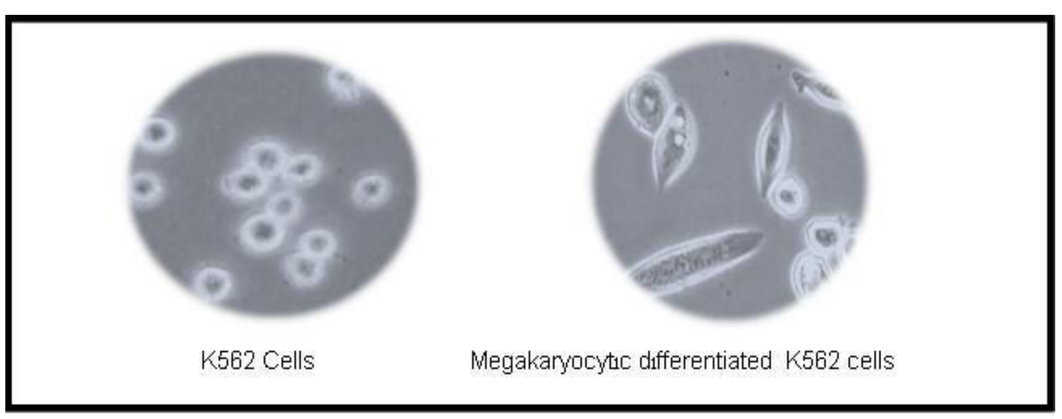

Figure 1. Effects of PMA, on K562 cell differentiation. K562 cells treated with 10nM PMA for 72 h . K562 cells (1 ×10 4cells/well) were cultured as described under material method. K562 cells were seeded in 96-well plates at a density of 10 4 cells/well in RPMI 1640 medium containing 1% fetal calf serum. Following 1 day of culture, differentiation was induced by the addition of 10 nM PMA for 72h. Megakaryocytic differentiation was observed by microscope.

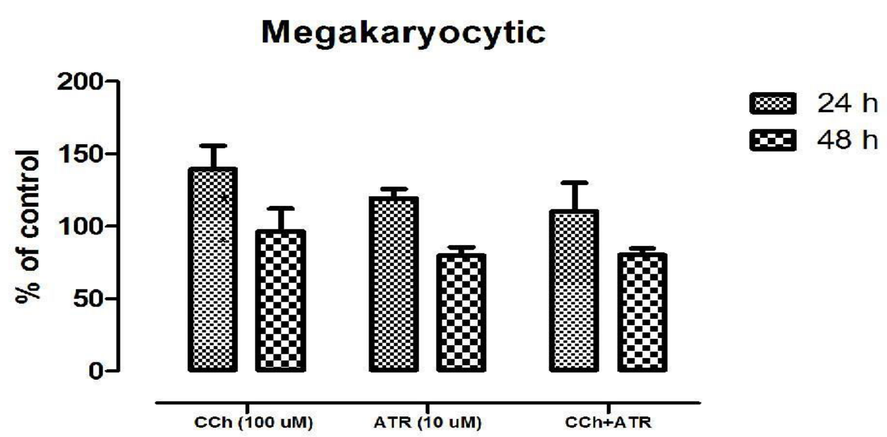

Figure 2. Effects of muscarinic agonist ,antagonist on megakaryocytic differentiated K562 leukemia cells proliferation. Megakaryocytic differentiated K562 Cells (1 × 10 4cells/well) were pre-treated with atropine for 30 min before the addition of CCh, as indicated. BrdU was applied for the last 4 h. Means ± SEM of n = 4 are shown. (* Significance of differences from CCh 24h alone ($P < 0.05$) .

Western blot analysis

We performed immunoblotting experiments in Megakaryocyte differentiated K562 cells. These cells were homogenized and Western blots were collected as shown in Figure 3. We observed in the expression of β3-integrin (Figure 3a).

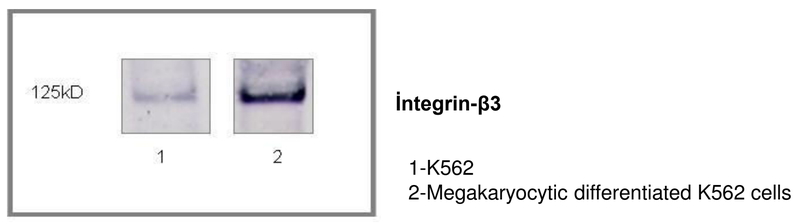

Figure 3. Megakaryocytic differentiated K562 cells express β3-integrin. Western blot analysis of integrin beta 3 in megakaryocytıc differentiated K562 cells. Western blot analysis of 100 µg total homogenates from megakaryocytıc differentiated K562 cells. The bands corresponding to integrin β3 in megakaryocytıc differentiated K562 cells. Molecular Weight was 125 kDa.

The expression of M_2, M_3, and M_4 receptors couldn’t be detected at the protein level by immunoblot analysis of the crude membrane fractions of megakaryocytic differentiated K562 cell lysates using specific, polyclonal antibodies.

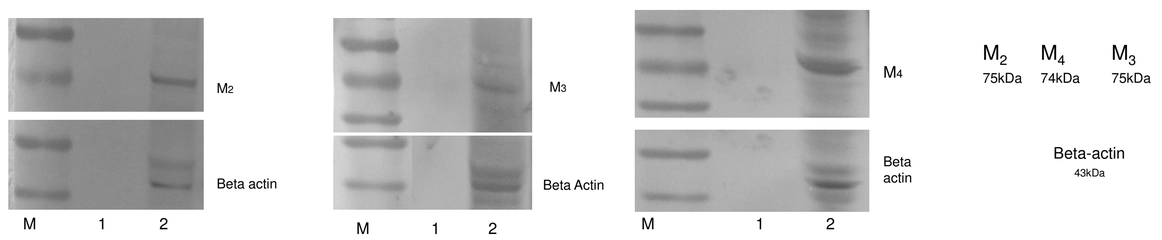

Figure 4: Western blot analysis of muscarinic receptor proteins (mAChR) in undifferentiated(1), megakaryocytıc differentiated(2)K562 cells. The bands corresponding to each muscarinic receptor subtype in K562 cells are shown. The estimated molecular weights were 75 kDa (M2, M3) and 74 kDa (M4). Blots are representative of three independent experiments.

We previously detected M2, M3 and M4 muscarinic subtypes in undifferentiated K562 cells (4). Expression M2, M3 and M4 (Figure 4) in cells. Extremely low level M3 expression detected in megacaryocyte differentiated cells.

Discussion

Chronic Myeloid Leukemia is frequently diagnosed more often in older adults. CML prevelance approximatly 15 percent of all leukemia cases(7). Human chronic myeloid cells express primarily M_2,M_3 and M_4 receptors (4). Our previous data showed that the proliferation of K562 cells in vitro largely depends on the presence of FBS. CCh treatment of serum-deprived K562 cells led to a significant increase in DNA synthesis [4,5). Activation of muscarinic receptors stimulates cell proliferation. Human chronic myeloid cells also produce and release acetylcholine at concentrations capable of activating M_3R and stimulating cell proliferation (Unpublished data) K562 cells have been used extensively to model for studying hematopoietic cell growth and differentiation (8). Different studies have suggested that muscarinic receptors also mediate some cellular events in hematopoietic cells. In this study, we examined whether cholinergic agonist /antagonist has an impact on proliferation to the PMA-induced megakaryocytic differentiated K562 cells . Upon exposure of PMA-induced megakaryocytic differentiated K562 cells supplemented with 1% serum to CCh, DNA synthesis was stimulated and this effect partly prevented by atropine. Thus, we demonstrated for the first time that carbachol enhances PMA-induced megakaryocytic differentiated K562 cell proliferation .

We detected changes in proliferation with carbachol in the PMA-induced megakaryocytic differentiated K562 cells . Our Western blotting data show that M_3 receptor protein expression very low level than control cells. We use same amount of protein lysate but we didn’t see effecient level protein bands on megakaryocytic differentiated K562 cells. However we must keep in mind that M_3 muscarinic receptor RNA level must be detected megacaryocytic differentiated K562 cells. These receptor may be useful as new therapeutic targets in Chronic Myeloid Leukemia.

Conclusions : We found that CCh changed proliferation of megakaryocytıc dıfferentiated K562 cells proliferation in 24 hour, These results suggest that CCh may be modulates megakaryocytıc dıfferentiated K562 leukemic cells proliferation through muscarinic acetylcholine receptors or nicotinic receptors.

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