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RESEARCH ARTICLE

Effects of filgrastim on gene expression of cholinergic system in the rat hippocampus

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ABSTRACT

Background: Filgrastim is a granulocyte colony-stimulating factor (G-CSF), which also showed effects on memory and cognation. Although recent studies have begun to explore G-CSF-related mechanisms of action in memory, little is known about its effects on cholinergic system. Since the cholinergic system has a key role in memory and some studies reported effects of G-CSF on cholinergic activity. Choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) gene expression was examined in the rat frontal cortex and hippocampus. **Aims and Objectives:** This study was designed to investigate the effect of G-CSF treatment on gene expression of AChE and ChAT in the frontal cortex and hippocampus during learning. **Materials and Methods:** In this study, male Wistar rats were treated for 2 week with filgrastim intraperitoneally at doses of 10, 50, 70 µg/kg/day. All rats received Morris water maze training (4 trials/day for 5 days). Moreover, ChAT and AChE gene expression was assessed by real-time polymerase chain reaction in the rat frontal cortex and hippocampus. **Results:** Over 5 days of training, filgrastim at doses 10, 50 and 70 µg/kg/day did not change the gene expression of ChAT and AChE in frontal cortex and hippocampus although at dose of 50 ug/kg a little changes but not significant observed. **Conclusions:** The findings observed in this study propose that G-CSF memory enhancer effects doesn't only cholinergic and other mechanism may be involve.

KEY WORDS: Acetylcholinesterase; Choline Acetyltransferase; Filgrastim; Granulocyte Colony-stimulating Factor

INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is a multimodal hematopoietic growth factor, which also has profound effects on the central nervous system (CNS).[1] G-CSF and its receptor have also been reported to be expressed in neurons and neural progenitor cells in the hippocampus and brain stem.[2] G-CSF passes the blood-brain barrier and possesses neuroprotective properties through interactions with G-CSF receptors.[3,4] Previous study indicated that G-CSF administration can ameliorate cognitive impairment in a Alzheimer disease (AD) model.[5] For the cognitive benefits of G-CSF, multiple mechanisms including reduction of inflammation, stimulation of neurogenesis and synaptogenesis and angiogenesis has been proposed.[5,6] The information currently available seems limited to the neuroprotective capabilities of G-CSF. The direct effects of G-CSF on the brain neurotransmitter system remain to be elucidated. The cholinergic neuron plays a very important role both in the central and peripheral nervous systems.[7] The central cholinergic deficit is strongly associated with some neurodegenerative diseases such as AD and Parkinson's disease.[8,9] Therefore, one of the most current treatments of AD is administrating acetylcholine esterase inhibitors such

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as tacrine and donepezil.[10] Choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) are specific cholinergic marker proteins for the functional state of cholinergic neurons, both of which can play a key role in the maintenance of ACh levels at the cholinergic neurons.[11] The frontal cortex and hippocampus were selected as the areas of interest in this study to see filgrastim effects on cholinergic function because these areas receive extensive innervation from cholinergic neurons in the basal forebrain.[11] The previous study indicated a great presence of AChE, ChAT, and the nicotinic and muscarinic acetylcholine receptors in the hippocampus.[12] The previous study showed that G-CSF was able to down-regulate inflammatory factor and up-regulate nicotinic AChR in the brain of AD model mice. They suggested that nAChR is essential for inhibiting inflammatory cytokine synthesis by the cholinergic anti-inflammatory pathway.[13] Other study also demonstrate that G-CSF, augment ChAT activity in primary cultured neurons and in cholinergic hybridoma cell line.[14] A more detailed understanding of the physiological role of G-CSF in the healthy brain may, however, open new insights into disease relevant mechanisms. This study was designed to investigate the effect of G-CSF treatment on gene expression of AChE and ChAT in the frontal cortex and hippocampus during learning.

MATERIALS AND METHODS

Adult male Wistar rats weighing approximately 250-300 g were used for the experiments. The animals were obtained from the GOUMS Animal Services. The animals were housed under controlled temperature and humidity with free access to food and water but food was not allowed 1 h prior of behavioral study and till its completion. All protocols for the experiments on animals were approved by the Research and Ethics Committee of Golestan medical Science University. Animals were randomly divided into three groups of 8 animal each including control (C) (untreated), and G-CSC. G-CSF was provided from Aria Tina Gen company (Gorgan, Iran) and after dissolving in saline administrated intraperitoneally at doses of 10, 50, 70 μg/kg to the rats for 2 weeks.

Learning

In the last week of injection, learning was made by the reference memory task in the Morris water maze (MWM). The MWM used in our study was a black circular pool (160 cm diameter, 60 cm high) filled with water (30 cm depth) at 24 ± 2°C. A submerged plexiglass platform (10 cm × 10 cm) was hidden 1 cm below the water surface and placed in a constant location in the center of Northwest quadrant. Animals received 5 days of training with the hidden platform, each day included 4 training sessions with a 60 s intersession interval. Each trial was started by placing a rat with its face toward the wall of the pool at one of three start points. The start location was varied on each training trial and changed each day. The trial was terminated when the animal entered the platform. If the rat did not find the platform within 60 s it was placed on the platform by the experimenter for 15 s. During acquisition of the spatial navigation task, all groups were given one session of four trials each day (day 1-5; trial 1-20).

Real-time Polymerase Chain Reaction (RT-PCR) Analysis

RNA was isolated from approximately 50 mg of frontal cortical and hippocampal tissues using the Gena bioscience RNA extraction kit according to the manufacturer's instructions. Residual DNA was digested with 10 U RNase-free DNase (DNase I, TaKaRa) in the presence of 20 unit of RNase inhibitor at 37°C for 20 min. After heat inactivation for 10 min at 75°C in 2 mM EDTA, total RNA solution was removed for quantification. Concentration and purity of the DNase I-treated samples was measured using a Nano Drop ND-1000 spectrophotometer (A260/A280>1.8 and A260/A230>1.6). The integrity and stability of the RNAs confirmed by demonstrating the intact 28 s and 18 s bands on gel electrophoresis. For RT-PCR, the cDNA was synthesized from 1 μg of DNaseI-treated total RNA using prime script RT reagent kit (Takara) with random hexamer and oligo dT primers following the manufacturer's protocol. The forward and reverse PCR primers for the 7 genes were designed accordance to the RT-PCR conditions, using perlprimer software (Bio-Rad, USA), and the sequences are listed (Table 1). For each gene, the cDNA amplified by specific primers using Taq Polymerase Kit (TaKaRa) and correct product was confirmed by running on gel electrophoresis (Table 1).

RT-PCR was performed using the SYBR-Green PCR Master Mix kit (TaKaRa) in the thermo Cycler (ABI, 7300). The cycling conditions were 95°C for 30 s followed by 40 cycles of 95°C for 5 s, 55°C for 30 s and 72°C for 1 min. We used rat β-actin as internal control and nondiabetic offspring islets cDNA as calibrator. Amplification specificity was confirmed by gel electrophoresis. The relative expression level of mRNA between the groups was determined with the comparative cycle threshold (CT) method. First, both CT values of target gene from control islets cDNA (n = 3) and diabetic islets cDNA (n = 5) were normalized by CT value of the internal control (ΔCT). Then, the former was subtracted by the latter, namely ΔΔCT. The value of 2 - ΔΔCT, the fold change of gene expression, was plotted into the figure. Every RT-PCR experiment was repeated with three samples and each sample was run in triplicate.

Data Analysis

Data were presented as mean±standard deviation. Relative target gene expression and blood glucose level was analyzed with one-way ANOVA using SPSS 16.0 statistical analysis

Table 1: Primers and their predicted sizes used for RT-PCR

Genes	Sequences 5=i3=	Size (bp)
β-actin	F-TCATGAAGTGTGACGTTGACATCCGTAAAGRCCTAGAAGCATTTGCGGTGCACGATGGAGG	285
AchE	F-GACTGCCTTTATCTTAATGTGR-CGGCTGATGAGAGATTCATTG	758
ChAT	F-GAGAAGACAGCCAATTGGGTCR-TTCATCCTCGTTGGACGCCAT	473

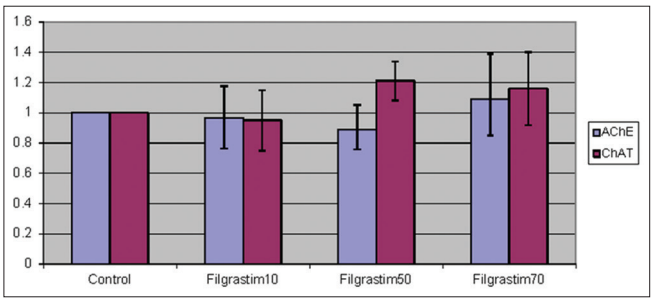

Figure 1: Real-time polymerase chain reaction analysis of choline acetyltransferase and acetylcholinesterase mRNAs, in the frontal cortex, mRNA levels were measured using gene-specific primers (Table 1) and the values were normalized to β-actin. Data are presented as mean ± standard deviation

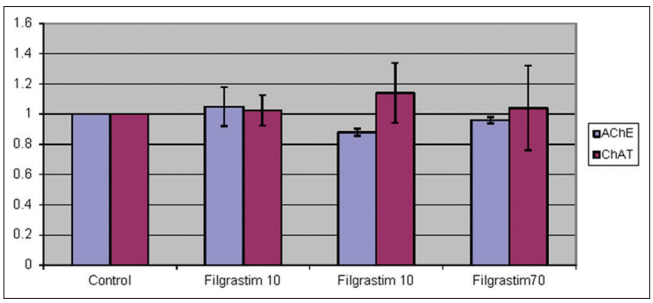

Figure 2: Real-time polymerase chain reaction analysis of choline acetyltransferase and acetylcholinesterase mRNAs, in the hippocampus, mRNA levels were measured using gene-specific primers (Table 1) and the values were normalized to β-actin. Data are presented as mean ± standard deviation

software. The differences between groups were compared using unpaired *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Representative RT-PCR of ChAT and AChE mRNAs, in the frontal cortex of rats treated with saline (control), filgrastim in dose of 10 and 50 µg/kg and 70 µg/kg are shown in Figure 1. Results did not show significant changes in expression of ChAT and AChE mRNAs in the filgrastim groups compared with the saline group ($P > 0.05$).

Representative RT-PCR of ChAT and AChE mRNAs, in the hippocampus of rats treated with saline (control), filgrastim in dose of 10 and 50 µg/kg and 70 µg/kg are shown in Figure 2. Results did not show significant changes in expression of ChAT and AChE mRNAs in the filgrastim groups compared with the saline group ($P > 0.05$).

DISCUSSION

There are now several lines of evidence suggesting G-CSF have different effect on cholinergic system. In this study, we examined the effects of G-CSF on of ChAT and AChE expression in the frontal cortex and hippocampus during learning. Animals were treated for 2 week with different doses of filgrastim and trained for 5 days in a reference memory task in the water maze. Rats treated with 10, 50 and 70 mg/kg/day of G-CSF did not show any changes in the gene expression of ChAT and AChE in frontal cortex and hippocampus. This was contrary to our expectation in our previous studies had observed that filgrastim enhanced the memory.[15] While other study indicated that *in vitro* granulocyte G-CSF augmented ChAT activity in mouse embryonic primary septal neurons and *in vivo* GM-CSF promoted survival of septal cholinergic neurons in adult rats which had undergone fimbria-fornix transections. The precise reason for this discrepancy is not known, but it is possible that the G-CSF acts in other ways. Previous studies on intracerebral hemorrhage indicated that beneficial effect of G-CSF on brain might be mediated through its anti-inflammatory effect.[16] In addition, it is possible that G-CSF memory enhancer effect was not cholinergic and another neurotransmitter may be involved.[17] Recently, it was demonstrated that G-CSF Increase ligand binding densities of NMDA receptors in hippocampus and enhanced the induction of long-term potentiation (LTP).[18] G-CSF has been known to have antiapoptotic effects in mature neurons and activates multiple cell survival pathways.[19] Both G-CSF and its receptors are widely expressed by neurons in the CNS,[2] and G-CSF can activate the transcription factor cAMP response element-binding protein (CREB) through the mitogen-activated protein kinase pathway. The CREB plays an important role for LTP, a cellular process associated with learning and memory.[20] Accordingly, G-CSF treatment improved spatial learning and reacquisition of information in rats engaged in a radial maze presumably by promoting the survival of new neurons,[18] which suggests an autocrine protective signaling mechanism that can improve memory in elderly.

CONCLUSIONS

Daily IP injections of G-CSF at doses of 10, 50 and 70 mg/kg for 2 weeks didn't change gene expression of ChAT and AChE in frontal cortex and hippocampus. We therefore, propose that G-CSF memory enhancer effect was not cholinergic and another mechanism may be involved.

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