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Original Article

Glia Maturation Factor Beta: A Novel Neuro-Impairment Prediction Factor in Toxoplasmosis

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Abstract

Background: *Toxoplasma gondii,* a neurotropic protozoan, infects up one to third of the world population. The parasite can invade a wide variety of nucleated cells but preferably glial cells. Glia maturation factor β (GMFβ), a 17KD protein expressed at high levels in the central nervous system is predominantly related to neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Multiple sclerosis. We aimed to determine the expression level of GMFβ and its relation to other pro-inflammatory factors (IL33, SDF1, and CCL2) on *T. gondii* infected human neuroblastoma cell line.

Methods: The human neuroblastoma (SK_NMC C535) cell line was infected by 5×10^6 (1:1 ratio). The supernatant was collected after cell lysis and centrifugation. Total RNA was extracted using the Yekta Tajhiz RNA extraction kit. cDNA was synthesized based on RevertAid First Strand cDNA Synthesis Kit manufacturer`s protocol (Parstous, cDNA synthesis kit, Iran). The specificity of each primer pair (GMFβ, IL33, SDF1, and CCL2) was provided by NCBI BLAST. Gene expression level was measured using Real-Time PCR. All experiments were conducted at the Hamadan University of Medical Sciences, western Iran in 2022.

Results: The GMFβ increased significantly up to 1.35-fold (P=0.007). The increase in GMFβ expression in neuroblastoma cells was consistent with the increase in pro-inflammatory factors (CCL2 (0.47), IL33 (0.152) and, SDF1 (1.33)).

Conclusion: GMFβ upregulation can be a novel indicator of the destruction of nerve cells.

Keywords: *Toxoplasma gondii;* Glia maturation factor; Interleukin-33; IL33; Chemokine CCL2; Stromal cell derived factor 1

Introduction

Toxoplasma gondii, a neurotropic protozoan, infects up one to third of the world population. Although infection in healthy immunocompetent individuals is asymptomatic, it may result in devastating neurological impairment via vertical transmission in the fetus or immunocompromised adults (1). Even though all organs can be involved by *T. gondii* tachyzoites in acute infection, the central nervous system is the most complicated part. According to

research, tachyzoites actively cross the blood-brain barrier over the early 7 d of infection (2, 3). The parasite in the brain can invade all types of nucleated cells and immunologically activate microglia and astrocytes. Cytokines and chemokines production within 7 to 10 days after infection, are reported followed by activation of glia to recruit peripheral T-cells and mononuclear cells (4). In transition of acute to chronic phase of infection, tachyzoites transform to bradyzoites while, the bradyzoites-containing brain cysts may become reactivated and lead to encephalitis (5). Encephalitis as a life-threatening infection has been reported in immunocompromised people due to the activation of parasitic cysts in the brain (6).

In the study of the parasite's preference in the invasion of nerve cells, the parasite can infect a large number of glial cells. Expression of Glia maturation factor β (GMFβ) has predominantly been reported from the central nervous system and glial cells. It is also expressed in other tissues including the thymus, testis, ovary, colon, and renal proximal tubule. (7, 8). Increased expression of GMF results in expression of immune-related mediators such as TNF-α, GMCSF and interleukin-1, associated with immunodegenerative diseases. The excessive expression in GMF can damage neurons (9). GMFβ may have both protective and deleterious effects on the pathogenesis and progression of various neuroinflammatory and neurodegenerative diseases such as Alzheimer's Disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) (10). It is not exactly clear which pathway is affected by the GMFβ, but the association between GMFβ and Cu-Zn superoxide dismutase (SOD), Granulocyte-macrophage colony-stimulating factor (GM-CSF), neurotrophins (NTs), as well as apoptosis is debated (11).

Interleukin 33 (IL33), as a candidate orchestra of immune cells, is involved in the pathology of brain tissue damage including, stroke (12, 13), neurodegeneration (14, 15), experimental autoimmune encephalomyelitis (EAE) (16, 17), and central nervous system (CNS) infection. IL33 receptor is also up-regulated in macrophages and astrocytes in the brain (4) and IL33/st2(receptor) signaling limit *T. gondii* growth and prevent neural damage but in large amount can have detrimental consequences (18). Oligodendrocytes and astrocytes were identified as main sources of IL33 in *T. gondii* infection. IL33 signals can promote production of CCL2 and CXCL10 (4).

Stromal cell-derived factor-1 (SDF1) is small chemokine, which activates the chemo taxis of immune cells and involved in pathogenicity of various diseases. However, the exact role of SDF1 is not fully understood in a healthy central nervous system; it is continuously expressed in neurons, glial cells, meningeal and endothelial cells (19-21). The interaction of SDF1 and its receptor are shown to lead to cell signaling including cell proliferation, differentiation, migration, survival, and apoptosis (22). The SDF1 was found to be increased over 24 h during focal cerebral ischemia by astrocytes, microglia, and vascular endothelial cells in damaged areas. SDF1 plays a role in the regeneration and reconstruction of neurons (23).

Chemokine (C-C motif) ligand 2 (CCL2) is a crucial element required for host resistance to the parasite. CCL2 expression is a common effector mechanism of innate parasite recognition (24). Researchers have shown that *T. gondii* infection is initially characterized by the expression of chemokines, especially CCL2, rather than cytokines such as interleukin 12. The onset of CCL2 production will be the beginning of an effective mechanism in *T. gondii* invasion (24).

We aimed to investigate the effect of *T. gondii* neuropathy on human neuroblastoma cell line as a human model of nerve cells by determining the expression level of GMFβ and its relationship with other pro-inflammatory factors (IL33, SDF1, and CCL2).

Materials and Methods

All experiments were conducted at the Hamadan University of Medical Sciences in 2022. One to two male Swiss albino mice were infected through intra-peritoneal injection of 10^3 *T. gondii* tachyzoites (25); after 3-5 d mice were euthanized by an overdose of ketamine/xylazine then mice were

submerged in 70% ethanol. Tachyzoites were harvested by flushing sterile phosphate-buffered saline (PBS; pH 7.3) into the peritoneal cavity in a hygienic condition. Following three times centrifugation at 1500 rpm for 10 min, tachyzoites were counted using a Neubauer hemocytometer at the concentration adjusted to 10^5 (26).

The human neuroblastoma (SK_NMC C535) cell line was purchased from Pasteur Institute Tehran, Iran. Cells were cultured in DMEM-12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/mL streptomycin using 25 cm2 flasks (Nunc, Denmark) and then were incubated in 5% CO_2. Purified *T. gondii* RH strain tachyzoites were added to 24-hour-old monolayer cell lines at the concentration of 50×10^6 (1:1 ratio). The viability of parasites was more than 99% (assessed by trypan blue exclusion), and there was less than 1% contamination by mouse cells. Media was replaced every 24 h. Number of tachyzoites, the viability of cell lines, and pH of media were investigated every day. Intact neuroblastoma cell line, as control, were treated by the PBS at the volume of 0.1 ml, simultaneously (26).

Neuroblastoma (SK-NMC C535) cells were infected up to 90% (after about 3 to 4 d). Following the spontaneous lysis of infected cell lines, the supernatant was harvested after 5 min centrifugation at 1500 rpm.

Total RNA was extracted using the Yekta Tajhiz RNA extraction kit. The quantity and quality of the RNA were checked using a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Madrid, Spain), in ratios between 1.9 and 2.1 in all the samples between A260/A280. The integrity of the RNA was verified on agarose gel electrophoresis stained with ethidium bromide.

cDNA synthesis was performed based on RevertAid First Strand cDNA Synthesis Kit manufacturer`s protocol (Parstous, cDNA synthesis kit, Iran) by incubation at 25 °C for 5 min, 42 °C for 1 h, and 70 °C for 5 min and stored at −80 °C for further analysis. Quantification of gene expression was followed by a Real-Time PCR system, BioRad CFX96, using RT^2 SYBR Green qPCR master mix according to manufacturer's protocol in 20 µl-volume including 1 µl cDNA, 7 µl H2O, 10 µl SYBR Premix Ex Taq II Kits (Takara, Japan), and 1 µl of 10 pmol/ml specific primer (GMFβ, IL33, SDF1, and CCL2).

Allele ID primers software was administered for primer design and precise validation. Checking of the specificity of primer pairs was provided by NCBI BLAST. Each PCR cycle consisted of an initial stage of denaturation at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 15 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec (27). Variation in gene expression level of samples was adjusted versus 18S rRNA as reference standard gene (28). Analysis of melting curves was conducted by using Light Cycler analysis when the amplification protocol was accomplished. The mean and SD of Ct values from the triplicate samples at each point were determined according to the ΔΔCT method. Normalization was done based on the internal control gene.

All statistical analysis was performed using SPSS ver.16 (Chicago, IL, USA). Variables were analyzed by Mann-Whitney and Kruskal-Wallis tests. All data was expressed as mean ± standard deviation (SD). The statistical level of significance was set at $P<0.05$.

Ethical approval

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. (Ethics committee code: IR.UMSHA.REC.1399.091).

Results

Forty-eight hours after cell infection, 80% to 90% of neuroblastoma cells were lysed. Real-time PCR was employed to determine the level expression of GMFβ, IL33, CCL2, SDF1 and 18SrRNA as a reference gene. Specific amplification was confirmed by the detection of a standard sigmoid melting curve and the absence of primer dimers or non-specific amplification (Fig.1). The validity of the

amplified products was finally confirmed by comparison of each sample with the DNA ladder according to the expected locus on a 2% agarose gel electrophoresis. The relative expression between the target genes to the reference sample was represented by calculation of delta threshold (ACT) value. Fold differences was expressed using the comparative 2–ΔΔCT formula. ΔΔCT values were increased up to 1.35 for GMFβ gene while it was significantly different versus control (*P*=0.007). Gene expression level for CCL2, IL33, and SDF1 in terms of fold difference by delta-delta-CT were up-regulated to 0.47, 0.152 and 1.33 respectively (*P*=0.007), (Fig.2).

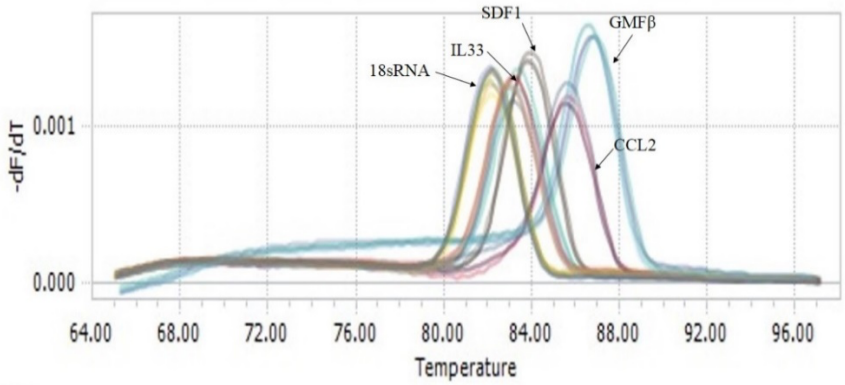

Fig. 1: Melting-curve analysis shows difference in melting temperature with single-peaked curves, indicating the specificity of the reaction and the absence of primer-dimer. Expression of GMFβ, IL 33, CCL2, SDF1, and 18 s rRNA reference gene by Real Time PCR

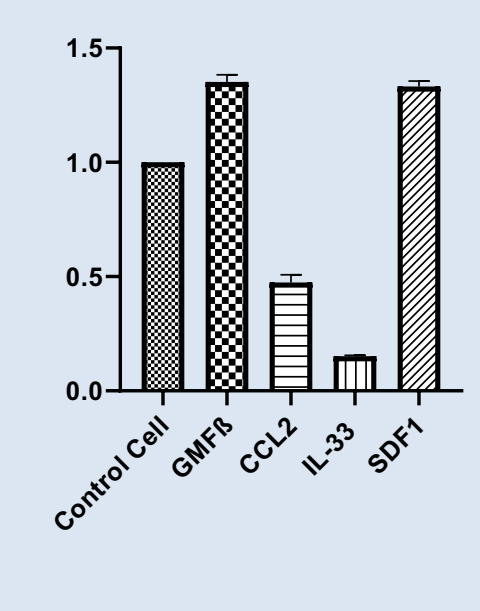

Fig. 2: Comparison of GMF-β gene expression, IL33, CCL2 and SDF1 in the infected cell line and control. The Real Time PCR reaction was performed twice on each gene and the mean ± SEM of gene folds were documented. Significant changes were found in genes expression folds in infected cells and control. **P*=0.007

Discussion

Neuroblastoma is a suitable cell line for investigating the neurodegenerative effect of damaging elements and infectious agents (29). Changes in the redox environment are related to the induction of brain pathological lesions in the Alzheimer's disease, and pro-oxidant factors are related to the amyloid/beta accumulation in the brains tissue (30, 31) in which antioxidant therapy improved lesions in the experimental model (32). Neurons are sensitive to the enhancement of oxidant agents, reactive oxygen species (ROS), reactive nitrogen species (RNS), and H2O2, which cause neuronal dysfunction, neuroinflammation, and cell apoptosis (33).

In our previous research, GMFβ as a neurodegenerative factor was significantly upregulated in the brain of toxoplasmic mice. It was followed by increased expression levels of CCL2, SDF1, and IL33 (34). Evidence has supported this idea that GMFβ leads to apoptosis and inflammation of nerve cells (9, 35). GMFβ can be a biomarker and a cell communicator alarm after damage at the early stages of a disorder (36).

GMFβ overexpression leads to the death of neurons because of neuroinflammation including AD, MS, and PD (37, 38). This can be proof of the relationship between neuroinflammatory diseases and chronic toxoplasmosis. Moreover, GMFβ is considered a new therapeutic target, so inhibition of expression can prevent the development of neurodegenerative diseases, including Alzheimer's (39).

Glia activation during inflammation leads to GMFβ and other cytokines and chemokines, oxygen, and nitrogen radicals' production, which brings out neuron inflammation and exacerbation the disease (40-42). Sun introduced GMFβ as a potential novel biomarker and therapeutic target. He showed that GMFβ expression was significantly higher than normal levels in tumor node metastases (TNM) stage and histopathological grade of hepatocellular carcinoma (HCC); thus, GMFβ gene knockdown with siRNAs reduced the proliferation, migration, and invasion in Hep3b cells (hepatocellular carcinoma) and stopped the expression level of metal matrix proteinases (MMPs) (43).

The previous in vivo study showed that the increased expression of GMFβ is in agreement with the expression of IL33, SDF1 and, CCL2 genes (34).

Several studies have identified the role of *T. gondii* infection as a risk factor for neurological and, behavioral disorders. Mental problems such as schizophrenia, bipolar disorder, depression and obsessive-compulsive disorder have been associated with human toxoplasmosis (44, 45).

According to Castano et al, a significant increase in IFNγ, TNF, CCL3, and CCL8 was identified on the 20th day of *T. gondii* infection (before neuroinflammation) compared to the control group. Cytokines and chemokines called for the migration of mononuclear cells to the central nervous system at the beginning of *T. gondii* infection and before the onset of neuroinflammation and mononuclear cells prevailed on d 30 and 60 (acute infection). However, during brain inflammation on day 45, the expression of IFNγ decreased while TNF remained high. Contrary to gene expression, the highest serum concentrations of cytokines and chemokines were observed on days 60 and, 90. CCLD2 and IFNγ also showed the highest concentration (46). The same results were recorded in which, significant increase of CCL2/MCP1 was observed in the brains of *Toxopalsma* infected wild type mice as compared to uninfected wild-type animals as well IFNγ$^{-/-}$ mice (47).

Immune mediators modulate Th1-dependent immunity as well as the anti-parasitic response during recruiting of mononuclear cells (48).

Based on Castano, behavioral changes in the experimental models of toxoplasmosis are regarded because of disturbed hemostasis of immunologic mediators in the brain tissue (46). IL33 is an inflammatory cytokine from the interleukin-1 family. In experimental ocular toxoplasmosis by Zhang et al.'s, the expression of the IL33 gene was increased in the eyes of Balb c and B6 mice (49).

Interleukin 33 is released from damaged or necrotic cells through the ST2 complex receptor, leading to immune system activation (50, 51). IL33 signaling is necessary to control brain *Toxoplasma* infection before encephalitis induction. ST2 receptor knockout BALB/c mice had higher parasite load (mRNA levels of BAG-1 (a bradyzoite specific antigen) and SAG-1 (a tachyzoite specific antigen) were 4.5 vs. 1 and 18 vs. 1.5, respectively) and much more intense cerebral lesions than the normal group. Accordingly, IL33 is intended to be called alarmin which is responding to parasite invasion earlier than other elements. (18). However, IL33 acts like a double-edged sword in inflammation. It switches on the immune system and intensifies the inflammation in overexpression. For example, the blockage of ST2 / IL33 mitigated the neutrophilic inflammation and suppressed and increased survival in C57BL/6 mice (52). The parasite virulence and the affected host tissue finely influence the dual role of IL33 whereas the further researches need to be carried out to determine the immunomodulatory role of IL33 and even its role as a therapeutic target (53).

Inflammatory mediators are regarded as fundamentals of innate and the development of acquired immunity. Cell damage is the first step for the initiation of immune cascade reactions during infection. Some of the cytokines or chemokines significantly increase during the acute phase of infection while others enhance in the chronic stage. Evaluation of cytokine gene expression before the onset of clinical manifestation as an alarmin will notify the CNS or eye involvement as soon as possible. Therefore, they can effectively prevent undesirable consequences or improve serious treat (54, 55). CCL2 and CXCL12 were introduced as biomarkers for the diagnosis of ocular toxoplasmosis in the chronic phase. In comparing the serum of patients with acute toxoplasmosis who had eye complications with healthy people, the interesting thing was that the expression of CXCL12 and CCL19 genes was higher in patients who had a larger necrotic lesion in the eye, in other words, measuring the expression of these chemokines, was a good target to determine the stage of the disease (56).

Conclusion

Human neuroblastoma SK_NMC C535 cell line was used as a useful model to investigate the neuronal injury; changes in the expression level of GMFβ in tachyzoite-infected neuroblastoma can be an appropriate biomarker of neurodegenerative damages in consequence of acute toxoplasmosis. Furthermore, the simultaneous increase of the expression of GMFβ and, IL33, CCL2, SDF1 reminded the alarming role of this biomarker to stimulate the expression of inflammatory cytokines and the beginning of pathological damages.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflicts of interest

The authors have no conflicts of interest.

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