Zinc increases nestin but not vimentin gene expression in mouse mesenchymal stem cells

Şefik Güran,[1] Zehra Dilşad Çoban,[1] Mualla Pınar Elçi,[1] Meral Sarper,[1] Ferit Avcu[1]

(1) University of Health Sciences, Gülhane Faculty of Medicine, Department of Medical Biology, Ankara, Turkey

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Corresponding Author:
Şefik Güran
University of Health Sciences,
Gülhane Faculty of Medicine,
Department of Medical Biology,
Ankara, Turkey
sefik.guran@sbu.edu.tr

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ABSTRACT

Objectives: Mesenchymal stem cells can differentiate into multiple cell lineages, including fibroblasts, adipocytes, chondrocytes, osteoblasts and neurons. Nestin plays an essential role in the central nervous system progression and in neurulation as an intermediate filament. Vimentin is an intermediate filament found generally in mesenchymal tissues. The expression of this gene is important in neuronal and glial differantiation. Zinc participates in many metabolic functions in body including mesenchymal stem cell differentiation. Here, the changes in nestin and vimentin gene expressions with zinc had been analyzed on mesenchymal stem cells. We found the role of zinc on these selected genes.

Methods: "Primary culture technique" was used in obtaining mouse mesenchimal stem cells in our laboratory. In identification of stem cells, colony forming was visualized and high gene expressions of specific genes were found. Zinc solutions in different concentrations were added to mesenchimal stem cell culture flasks. RNA isolation and c-DNA synthesis were performed for each flask. Nestin and vimentin gene expression changes were found by using real-time polymerase chain reaction in uses of zinc in different concentrations.

Results: Due to these results, zinc increased the expression of nestin gene on mesenchymal stem cells. No stimulatory and/or inhibitory effect of zinc was found on vimentine gene.

Conclusions: High nestin gene expression probably represents the role of zinc on the mesenchymal stem cell differentiation via nestin gene. Zinc hasn't inhibitory and stimulator role on vimentine gene differantiation. Zinc increases nestin but not vimentin gene expression in mouse mesenchymal stem cells.

Introduction

Mesoderm differentiates into blood, bone, muscle, cartilage and fat tissues in development of vertebral embryos (1). Mesenchymal stem cells (MSCs) are mesodermal stem cells found in adults (2). Recent studies suggest that MSCs differ into neurons also under appropriate conditions. MSCs co-cultured with cerebellar granule neurons have been shown to differentiate into a neuron. This new regenerated neuron cell expresses nestin protein (3,4). Nestin protein (Neural stem cells protein) is an intermediate filament that plays an essential role in central nervous system progression and neurulation (5). Nestin is related with "embryonic and induced pluripotent stem cell differentiation pathways" and "neural stem cell differentiation pathways" as a lineage-specific marker (6). Nestin (NES gene; OMIM NO: 600915) gene expression is required for survival, renewal and mitogen-stimulated proliferation of neural progenitor cells (1). Vimentin (VIM Gene; OMIM NO; 193060) gene encodes other type of intermediate filament protein. Intermediate filaments, along with microtubules and actin microfilaments, make up cell cytoskeleton. The encoded protein is responsible for maintaining cell shape and integrity of cytoplasm. Vimentin as a protein is involved in neurogenesis. Vimentin acts as an organizer in cell attachment, migration, and signaling. Generally it exists in non-epithelial cells, such as mesenchymal cells (7). Despite of these facts, the factors that alter gene expressions of nestin and vimentin genes in cell differentiation are not fully known. Zinc (Zn) is an important element that participates in many metabolic functions in a cell. It changes gene expressions and alters the functions of these genes (8, 9). So, the possible roles of Zn on nestin and vimentin genes were studied in our experiments on mouse MSCs. In our study, Zn (in 8 µl, 16 µl, 32 µl, 64 µl and 128 µl concentrations) were applied in mouse MSCs. Total RNA isolation and c-DNA synthesis were performed in

each culture. Real Time Polymerase Chain Reaction (RT-PCR) analyses were performed for finding the expressions of nestin and vimentin genes. It was found that, Zn increased nestin (but not vimentin) gene expression in Zn treatment on mouse MSCs. This finding can be interpreted as the ability of Zn to be used in differantiation MSC in certain pathways.

Methods

Mouse mesenchymal stem cell obtaining from primary culture of mouse bone marrow;

"Primary culture technique" was used in obtaining mouse MSCs in our laboratory. MSCs were received from primary bone marrow cultures of a 'balb-C' mouse by using Coban et al. methodology (The ethics committee decision-Gülhane Military Medical Academy/Ethics-2012-4) (10). Mouse pharyngeal femurs were sacrificed by cervical dislocation under anesthesia. The femurs removed from joints were cleaned from soft tissues. The joints were cut by a surgical blade. Bone marrow was removed by cutting both joints in culture medium (RPMI-1640 Medium-Sigma Aldrich/ R8758). Culture medium was injected with insulin injector to the interrupted bones. The cells in culture medium were collected carefully. Bone marrow cells obtained from mouse femur were centrifuged twice for 5 minutes at 1000 rpm. Supernatant of bone marrow was discarded. Underlying cells were collected from the bottom of falcon tube. Culture medium (10 ml RPMI) was added again. Cells were incubated in 25ml culture flasks (Thermo Scientific Heraeus Incubator). Three days later, the floating cells were discharged with medium. The cells attached to the bottom of culture flasks are MSCs according to Zhang et al.'s methodology (10, 11). The cells were harvested by using trypsin/EDTA solution (Sigma Aldrich/ T4049). "Colony forming" in cultured cells is specific form for a stem cell colony (10, 12). In identification of MSC, "colony forming" was observed in culture flask under inverted microscope (Zeiss AX10 inverted microscope). Cell receptors which are specific for MSCs were analyzed in our cell line (CD 73, CD 90 and CD 105) (10, 13). High expressions of CD 73, CD 90 and CD 105 genes were found in our cells. Negative CD34 gene expression was observed in RT-PCR analyses (10, 13).

Preparation of Zn solution and application to cell cultures;

Zinc chloride (ZnCL2, SIGMA Cat No: Z0152) stock solution was prepared by dissolving in 30 mM HCl to obtain a 10 mM stock solution by using Zemann et al's protocol (14). Different Zn solutions were prepared in 8 µM, 16 µM, 32 µM, 64 µM and 128 µM concentrations. The control group (untreated with Zn) and five flasks (treated with Zn) were prepared in our study. The cultured cells were treated with 8 µM, 16 µM, 32 µM, 64 µM and 128 µM Zn solutions. In control group, no Zn treatment was applied. Zn treated culture results were correlated with the control culture result.

XTT Cell Proliferation Assay;

The cytotoxic effects of Zn solutions were analyzed by using the protocol of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl)]-2H-tetrazolium hydroxide) assay kit on mouse MSCs. "XTT assay" kit (Trevigen XTT Cell proliferation assay kit-Cat No: 4891-025-K) was used in accordance with manufacturer's instructions. The results were obtained by using "ELISA reader" (15). XTT assay results revealed that Zn solutions used in our study are below the toxic dose.

RNA Isolation from cell culture;

Cells were harvested by using trypsin/EDTA solution (Sigma Aldrich/ T4049) 24 hours after Zn application. RNA isolation was performed three times in each culture flasks (For analyzing the expression of nestin and vimentin genes). The c-DNAs were obtained in each condition. The c-DNAs were run on 2% agarose gel for control.

Reverse transcription polymerase chain reaction (RT-PCR) analyses;

The obtained c-DNAs were used as a template for PCR and RT-PCR. Nestin gene primers used in PCR [5'-CCC TGA AGT CGA GGA GCT G-3 '(forward) and 5'-CTG CTG CAC CTC TAA GCGA-3' (reverse)] and Vimentin gene primers used in PCR [5'-CGTCCACACGCACCTACAG-3' (forward) and 5'-GGGG-GATGAGGAATAGAGGCT-3' (reverse)] were taken from website (http: //mgh.harvard.edu/primerbank/). C-DNA fragments were obtained in RT-PCR by using general conditions (Tm, 62 degrees, 30 cycles). Beta-actin gene was used as an internal control (16). RT-PCR mix [10 µl of SYBR, 5 µl of DNA, 1 µl of primer, 4 µl of dH 2 O] was prepared as 20µl in each reaction (repeated 3 times). Based on the results obtained by RT-PCR, a correlation was analyzed in between nestin and vimentin gene expressions and Zn concentrations.

Statistical analysis of RT-PCR results;

Standard deviations (SD) values were obtained by taking arithmetic averages in the obtained values. The results were evaluated by Student's t-test to find p-values.

Result

In identification of MSCs, we observed "colony forming" in primary cell culture as described in a previous manuscript (10). Gene expression analyses results revealed positive CD 73, CD 90, CD 105 and negative CD34 (10, 11).

In RT-PCR analysis in control group (untreated with Zn), nestin gene expression level was found to be 1.32 ± 0.6 in average. Nestin gene expression levels were found as 1.28±0.1 and 1.34±0.4 in treated 8 µM and 16 µl Zn concentrations respectively. These values obtained in Zn treated groups were found to be statistically insignificant when compared with the control group ($p \geq 0.05$) (Table 1). At 32 µM, 64, and 128 µM concentrations, nestin gene expressions were analyzed as 1.42±0.3; 2.10±0.2 and 2.22±0.2 respectively. These values were statistically significant when compared to the control group (untreated with Zn) ($p<0,05$) (Table 1). This result should be interpreted: $p<0.05$ that Zn significantly increased nestin gene expression at high concentrations (32 µM, 64 µM and 128 µM) (Table 2).

As seen in table 1, vimentin gene expression was found as 2,46±0,9 in control group. It was found as 2.40±0.4; 2.45±0.4; 2.43±0.9; 2.48±0.7 in 8 µl, 16 µl, 32 µl, 64 µl and 128 µl Zn treatments respectively (Table 1). These values were found to be statistically insignificant when compared with control group (untreated with Zn) ($p \geq 0.05$) (Table 1). According to this result, Zn had no effect on vimentin gene expression (in concentrations of 8 µl, 16 µl, 32 µM, 64 µM and 128 µM Zn) (Table 2).

Discussion

MSCs are the adult stem cells that originate from mesoderm. MSCs take place especially in stromal tissues. In certain conditions, MSC can differentiate to other kinds of mesenchimal cell such as osteocytes, chondrocytes or adipocytes. In appropriate

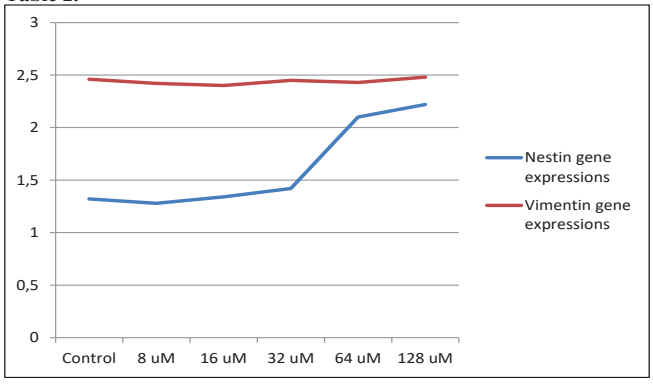
Table 1.

	Zn concentrations										
	Control	8 µl		16 µl		32 µl		64 µl		128 µl	
			p		p		p		p		p
Nestin gene expressions	1.32±0.6	1.28±0.1	≥0.05	1.34±0.4	≥0.05	1.42±0.3	<0.05	2.10±0.2	<0.05	2.22±0.2	<0.05
Vimentin gene expressions	2.46±0.9	2.42±0.1	≥0.05	2.40±0.4	≥0.05	2.45±0.4	≥0.05	2.43±0.9	≥0.05	2.48±0.7	≥0.05

conditions in laboratory, they may also transform into non-mesenchymal cells such as neural cells (1, 2). Bone marrow is the original source of MSCs, and still is the most frequently utilized. These bone marrow stem cells do not contribute to the formation of blood cells. They do not express the hematopoietic stem cell marker CD34 (17). These cells displayed a stable phenotype and remained as a monolayer in vitro (18). Here we isolated mouse MSCs in our laboratory under appropriate conditions. These cells were characterized by "colony forming" in cell culture (10-12). In mouse and human MSCs generally express special markers including CD73, CD90 and CD105. Unlike the other bone marrow cells, MSCs do not express CD34 (11). Here, we observed in RT-PCR analyses that CD73, CD90, CD105 positivity and CD34 negativity in our MSCs in standardization of MSCs (10).

Conversion of MSCs into functional neurons is important in terms of regenerative therapy approaches. Significant studies have been carried out in literature in recent years (3, 19). In neuronal differentiation, nestin gene expression is essential. In nestin suppressed condition, no differentiation occurs in neuronal stem cells (4). Nestin also takes place on embriyonal stem cell differantiation. Nestin as an intermediate filament first identified in neuroepithelial stem cells, is expressed in migrating and proliferating cells in embriyonal development. Increase in nestin gene expression in co-cultures with other cells reveals cell to cell interaction (20). Vimentin is another intermediate filament protein especially in glial cells in brain (21). Early during development of glia and immature astrocytes express mainly vimentin. Early during development, radial glia and immature astrocytes express vimentin mainly. Towards the end of gestation, a switch occurs whereby vimentin is progressively replaced by glial fibrillary acidic protein-GFAP in differentiated astroglial cells. The expression of vimentin and GFAP increased markedly after injury to central nervous system (22, 23). So, nestin and vimentin gene expressions were analyzed in our study on mouse MSCs obtained in our laboratory.

Table 2.



Zn is an important trace element in the structure of hundreds of enzymes in the organism. They have roles in the functions of the central nervous system from early embryogenesis to adult turnover. Zn regulates gene expressions of various transcriptional factors at the molecular level. It is involved in the structure of key enzymes necessary for neuron metabolism (24). At the cellular level, Zn is a modulator of synaptic activity and neuronal plasticity. Also, Zn plays a key role in signal transduction and neurotransmitter activity (25). Despite of these findings, Zn metabolism is not clear in differentiation of MSCs. In our study, Zn functions were analyzed on mouse MSCs with the expression findings of nestin and vimentin genes which are important in differentiation of MSCs (26). Zn increased the gene expression of nestin (but not vimentin) on MSCs in our study (Table 1 and 2). Zn effects on nestin gene directly in MSCs in our study. This finding may represent that Zn causes MSC differentiation using some pathways which affects on nestin gene. In literature, no similar findings were found on Zn and nestin gene expression on MSCs. Shao et al. were applied 50 and 100 µM Zn on Caco-2 cells in a recent manuscript. They found that Zn enhances intestinal epithelial barrier function through the PI3K/AKT/mTOR signaling pathway in 100 µM Zn treatment (27). Liu et al. explained that nestin over expression promotes the embryonic development of heart and brain through the regulation of cell proliferation. They noticed that nestin over expression is responsible for a marked activation of the PI3K/Akt/mTOR signaling pathway (28). In other manuscript, nestin reported as an essential protein for mitogen-stimulated proliferation of neural progenitor cells. In this manuscript, the relationship among nestin and PI3K receptor protein was explained clearly. Nestin gene product as described an essential protein for the proliferation of neural progenitor cells by promoting the activation of PI3K (29). These findings probably an evidence for a stimulatory affect of Zn on nestin gene. Nestin gene over expression affects stem cell differantiation on PI3K/Akt/mTOR signaling pathway. Yang et al. reported that PSMB8 (a therapeutic target for glioma treatment) decreased vimentin gene expression. The same molecule induces apoptosis of glioma cells via upregulation of caspase-3 expression. PSMB8 uses ERK1/2 and PI3k/AKT signaling pathways in cell apoptosis (30). Wu et al. reported miR-125b role on the differentiation of MSCs into neuron-like cells. They looked for the role of nestin and vimentin genes at the same time on this process. They found only the role of nestin protein. So, affected mechanisms in nestin and vimentin genes are different in some situations due to literature (31). In our experiment Zn affected only nestin gene expression by using probably a different pathway in MSCs.

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Conflict of Interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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