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Anti-cancer effects of Staphylococcal Enterotoxin type B on U266 cells co-cultured with Mesenchymal Stem Cells

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ABSTRACT

Background: Malignant plasma cells are responsible for Multiple Myeloma (MM). Myeloma Cells (MCs) are located in Bone Marrow (BM) and are in contact with stromal cells. The BM-derived Mesenchymal Stem Cells (BM-MSCs) affect MCs biology through different mechanisms. Currently, Staphylococcal Enterotoxin B (SEB) has been introduced as an anti-tumor agent that is able to kill cancer cells. The present study examined the effects of SEB on MCs and MSCs as an anti-tumor substance.

Methods: U266 cells co-cultured on BM-MSCs and treated with SEB and cell viability was analyzed by MTT assay and flow cytometry. The expression levels of IKKb, IL-6, IL-10, and TGF-β genes were evaluated by Real Time-PCR technique in U266 cells and BM-MSCs.

Results: Data showed that in the presence of SEB, BM-MSCs support U266 cells proliferation and survival. Moreover, SEB, BM-MSCs and BM-MSCs Conditioned Medium (CM) up-regulated IL-6 and IL-10 expression in U266 cells. Additionally, U266 cells showed increased levels in IKKb expression in presence of SEB or BM-MSCs, while expression of IKKb in U266 cells was down-regulated in coexistence of SEB with BM-MSCs or SEB with CM. Also, TGF-β remained without any changes.

Discussion: All in all, SEB can be an appropriate candidate to decrease proliferation and survival rate of cancer cells and it can make noticeable alteration in expression of some genes in U266 cells and BM-MSCs. Further molecular studies are needed to identify the mechanism of action of SEB on U266 cells and BM-MSCs.

1. Introduction

Plasma cells are one of the most critical members of Bone Marrow (BM) milieu that are important in hematopoiesis and lymphopoiesis. Multiple Myeloma (MM) is a plasma cells malignancy which can affect many organs and is characterized by plasma cells accumulation in BM, bone erosion, suppression of hematopoiesis, and immune system deficiency [1,2]. Plasma cells accumulation in BM is a cytological sign that confirms the supportive roles of BM microenvironment [3]. The BM microenvironment contains several components including stromal cells. In this regard, recent studies made numerous efforts to recognize the stromal cells impressions on Myeloma Cells (MCs), because MCs and stromal cells reciprocally trigger different biological signaling pathways [4]. Mesenchymal Stem Cells (MSCs) are one of the BM stromal cells and they are multipotent stem cells. MSCs are found in different types of tissues such as adipose, cartilage, muscle, and BM. MSCs serve as a pivotal component for normal and malignant cells in BM milieu [5–7]. Given this fact, several studies have been conducted on MCs and MSCs issue [8,9]. In more details, MSCs can influence survival, invasion, and drug resistance of MCs via changes are made in signaling pathways [4].

As it is known, Nuclear Factor-kappa B (NF-κB) pathway is over-activated due to some mutations in MCs. Furthermore, MSCs enhanced exaggerated NF-κB signaling pathway activity in MCs. NF-κB signaling pathway is responsible for different biological mechanisms such as proliferation, drug resistance, and invasion in MCs. Indeed, several studies confirmed that NF-κB signaling pathway is involved in Interleukin-6 (IL-6), Interleukin-10 (IL-10), and TGF- β production. In MCs, these cytokines lead to an enhancement in proliferation rate, immune system suppression and malignant plasma cells growth, respectively [10–16]. In this regard, suppression of these cytokines with minimum side effects on patients with plasma cells malignancies can be in favor of researchers and clinicians. Hence, there is a necessity to explore more efficient drugs.

Our early studies showed that Staphylococcal Enterotoxin B (SEB) is

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Abbreviations

BM	Bone Marrow
MM	Multiple Myeloma
MCs	Myeloma Cells
SEB	Staphylococcal Enterotoxin B
NF-κB	Nuclear Factor-kappa B
IL10	Interleukin 10
IL6	Interleukin 6
CM	Conditioned Medium

a potent agent for inducing apoptosisin different types of cancer cells through various axises [17,18]. On the other hand, SEB showed proliferative potential for $CD4^+$ and $CD8^+$ T cells subtypes. Additionally, it is demonstrated that the levels of Th1-derived cytokines including IL-2, TNF-α, and IFN-γ increased in hyperactivation of T cells [19,20]. Based on proved effects of SEB on immune system function, we investigated the probably therapeutic effects of SEB on U266 cells and BM-MSCs, for the first time. To this purpose, we simulated a co-culture system and analyzed proliferation rate, viability of U266 cells on BM-MSCs. With respect to the importance of NF-κB pathway in MM, we analyzed IKKb, as a key molecule in this pathway and the production of some other related genes in U266 cells and BM-MSCs were investigated.

2. Materials and methods

2.1. Cells culture

The BM-MSCs were obtained from Pasteur Institute of Iran and were cultured in Low Glucose Dulbecco's modified Eagle's medium (Low Glc-DMEM) (Gibco Invitrogen, Karlsruhe, Germany) with 10% Fetal Bovine Serum (FBS) (Gibco Invitrogen, Karlsruhe, Germany) and 1% penicillin and streptomycin (Sigma-Aldrich, Schnelldorf, Germany). Then, BM-MSCs were incubated in a humidified incubator (MEMMERT® incubator, Bellevue WA 98005, US) at 37 °C and 5% CO_2. Cell's medium was replaced every two days. U266 cell line (Human Multiple Myeloma cell line) was purchased from Pasteur Institute of Iran. U266 cells were grown in RPMI-1640 (Gibco Invitrogen, Karlsruhe, Germany) with 10% FBS (Gibco Invitrogen, Karlsruhe, Germany) and 1% penicillin and streptomycin (Sigma-Aldrich, Schnelldorf, Germany). Then, U266 cells incubated in at 37 °C in 5% CO_2 and 95% air. The medium was changed every day and Trypan Blue (Sigma-Aldrich, Schnelldorf, Germany) staining was used for cell viability assay.

2.2. Optimum concentration of SEB

U266 cells (10^5 cell/well) were cultured in a 96-well plate with RPMI-1640 and 10% FBS for 24 h. Then, different concentrations of SEB (Sigma-Aldrich, Schnelldorf, Germany) (25 μM/ml, 35 μM/ml, 45 μM/ml, 55 μM/ml and 65 μM/ml) were added to U266 cells and were incubated for 48 h. The MTT assay was performed to determine the effect of various concentrations of SEB on U266 cell proliferation. MTT assay showed that 35 μM/ml of SEB was appropriate concentration for our purpose.

2.3. Conditioned Medium (C.M) preparation

The BM-MSCs were cultured in DMEM medium and incubated to reach to 60–70% confluency. The adherent BM-MSCs were treated with 35 μM/ml of SEB for 48 h. In the next step, the culture medium was replaced with RPMI-1640 without FBS and BM-MSCs were incubated for 72 h. Then, the medium was collected, cells were removed by centrifugation, and the supernatant was concentrated by 10 kDa MW cut-off ultrafiltration membranes (Sigma-Aldrich, Schnelldorf, Germany) [21]. For sterilization, 220 nm filters (Sigma-Aldrich, Schnelldorf, Germany) were used. To treat cells, sterilized C.M was diluted with PBS (Sigma-Aldrich, Schnelldorf, Germany), at 250 fold, and protein concentration was reached to 46.3μg/μlThe Bradford assay was used to measure protein concentration.

2.4. Proliferation assay

First of all a co-culture system was designed. To this purpose, BM-MSCs were seeded in different densities (5×10^3, 1×10^4, 2×10^4 and 5×10^4 cell/well) in 6-well plates (Orange Scientific, Braine-l'Alleud, and Belgium), then U266 cells were added (1×10^5 cell/ml). MTT assay performed after 48hrs.

In the next step, to investigation of SEB effects on U266 cells proliferation we cultured U266 cells (1×10^5 cell/ml) in four groups: 1) U266 cells in RPMI-1640 as a control group, 2) U266 cells with SEB (35 μM/ml), 3) U266 cells with SEB (35 μM/ml) and BM-MSCs, and 4) U266 cells with SEB (35 μM/ml) and CM. Then, we incubated cells for 24, 48, 72, and 96 h. After incubation, MTT assay was performed.

In brief, 100 μl MTT solution (Sigma-Aldrich, Schnelldorf, Germany) was added to each well and the plates were incubated at 37 °C. Then, the DMSO (Merck, Germany) treatment was performed and the solution was left for about 2.5 h. At the end, the solution was clarified by centrifugation and the absorbance was measured at 580 and 650 nm by ELISA reader (Stat Fax 3200, Awareness Technology Inc, USA).

2.5. Detection of viable cells by Annexin V/PI double staining assay

U266 cells (1×10^5 cell/ml) were cultured with BM-MSCs (1×10^4 cell/ml, confluency 60–70%), C.M, and RPMI-1640. After 24 h incubation, SEB (35 μM/ml) was added to each group and remained for 48 h. Approximately, 1×10^5 U266 cells were harvested followed by a washing step using PBS. After centrifugation, 500 μl of 1X binding buffer was added to U266 cells pellet. In the next step, 5 μl of Annexin V-FITC (Abcam, USA) (100 ng/ml) and 5 μl of propidium iodide (PI 50 μg/ml) (Abcam, USA) were mixed with the mixture of cells and binding buffer. The solution was incubated at room temperature for 5 min in dark. At last, samples were analyzed by flow cytometry (FACS Calibur, BectonDi-ckinson, USA).

2.6. Gene expression

For quantification of genes expression, U266 cells (1×10^6 cell/ml) were cultured in five groups as follows: co-cultured U266 cells with BM-MSCs, treated U266 cells with SEB (35 μM/ml), cultured U266 cells in C.M, co-cultured U266 cells with BM-MSCs and SEB (35 μM/ml), and cultured U266 cells in C.M supplied with SEB (35 μM/ml). After 48 h incubation, 1×10^6 cell/ml U266 and 1×10^6 cell/ml BM-MSCs were harvested separately, washed by centrifugation, total RNAs were extracted by RNA isolation kit (Yekta Tajhiz Azma, Iran), and reverse transcription of mRNA to cDNA was done bycDNA synthesis kit following manufacturer's instruction (Yekta Tajhiz Azma, Iran). Real time-PCR reaction was performed using SYBER Green PCR Master Mix kit (Yekta Tajhiz Azma, Iran) and the used primers (primers was designed with primer3 software) are as follows: 5′-ACAGCGAGCAAACCGAGTT TGG-3′ and 5′-CCTCTGTAAGTCCACAATGTC GG-3′ for human IKKB; 5′-AGACAGCCACTCACCTCTTCAG-3′ and 5′-TTCTGCCAGTGCCTCTTTG CTG-3′ for human IL-6; 5′-TCTCCGAGATGCCTT CAGCAGA-3′ and 5′-TCAGACAAGGCTTGGCAACCCA-3′ for human IL-10; 5′-TACCTGAA CCCGTGTTGCTCTC-3′ and 5′-GTTGCTGAGGTATCGCCAGGA A-3′ for human TGF-β; 5′-TGTCTTCACCACCATGGAGAAGG-3′ and 5′-GTGGAT GCAGGGATGATGTTCTG-3′ for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (TAG Copenhagen, Denmark). Gene amplification was performed according to the following PCR program: 95 °C for 3 min and 1 cycle, 95 °C for 10 s and 40 cycles, 53 °C for 15 s, 72 °C