doi.org/10.22037/aab.v11i2.31055

Original Article

The Effects of Conditioned Medium from Bone Marrow-derived Mesenchymal Stem Cells on EMT Markers

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Article Information
Received:2020-6-22
Revised:2020-6-30
Accepted:2020-07-01

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Cite this article as:
The Effects of Conditioned Medium from Bone Marrow-derived Mesenchymal Stem Cells on EMT Markers. Mirzaei S, Baghaei K, Parivar K, Hashemi M, Asadzadeh Aghdaei H. Archives of Advances in Biosciences 2020:11(2)

Abstract

Introduction: Epithelial-Mesenchymal Transition in colorectal cancer cell is a critical process in which cells lose their epithelial properties and obtain mesenchymal characteristics, resulting in tumor cells and metastasis. This study attempted to work on the effects of conditioned medium from bone marrow-derived mesenchymal stem cells on EMT markers.

Materials and Methods: In this study, HT29 was used which is the colorectal cancer cell line. Cells were treated for 72 hours with BMSC-CM in order to induce EMT in HT29. The Real-Time PCR was used for evaluating EMT markers such as E-cadherin- β-catenin -vimentin-and transcription factors.

Results: Inducing EMT in colorectal cancer cells caused morphological changes. It was manifested that E-cadherin is downregulated after induction of EMT with treated BMSC-CM. On the other hand, there were a significant increase in β-catenin, Vimentin, Snail and ZEB1 expression.

Conclusion: Understanding the molecular basis of tumor metastasis is critical for colorectal cancer treatment. Findings demonstrated morphological alterations in consequence MSCs-CM activates induction of EMT. This process affects EMT markers of E-cadherina, Vimentin, β-Catenin and transcription factors of Snail and ZEB1. This model helps knowing cancer and metastasis pathway and also could be used in drug screening procedures.

Keywords: Cancer Stem Cells, Colorectal Cancer, Epithelial-Mesenchymal Transition, Conditioned Medium, Bone Marrow Mesenchymal Stem Cell

1. Introduction

Metastases pose a therapeutic challenge for the treatment of colorectal cancer (CRC), the third most common cancer and the second leading cause of cancer-related deaths in the United States, with an estimated 140,000 new cases and 51,000 deaths in 2018 [1]. Approximately 21% of CRC patients will have distant metastases and, despite favorable results for localized and regional diseases (89.8% and 71.1% respectively 5-year survival), metastatic disease 5-year survival is around 14% [2]. However, 50% of metastatic disease patients have liver-only disease that can be amenable to therapeutic intervention [3]. Nevertheless, conventional therapy is not appropriate for most metastasized CRC patients [4, 5]. In other words, advanced CRC with metastasis does not have an effective therapy [6]. To sum up, it is important to find new biomarkers for CRC diagnosis and establish novel treatment

methods [7]. Metastatic cancer development involves alterations in epithelial structure and function [8]. In other terms, numerous studies have shown a direct relationship between metastasis and the phenomenon of Epithelial-mesenchymal transition (EMT) [9]. Recent evidence suggests the epithelial-mesenchymal transition (EMT) promotes migration, invasion, and metastasis of tumor cells [10, 11]. It is widely accepted that a fundamental process for distant metastasis formation is the epithelial-mesenchymal transition (EMT), during which tumor cells lose their epithelial properties and acquire a fibroblast-like phenotype [12, 13] by reducing the expression of epithelial markers such as E-cadherin and the expression of mesenchymal markers such as Vimentin [14]. Signals facilitating and controlling this process in developmental and cancer EMT is derived from a microenvironment of the tissue and typically function as transcriptional repressors. They are also associated with zinc-finger factors including Snail1, Snail2 (Slug), ZEB 1/2 and Twist 1/2 baseline helix-loop-helix proteins (bHLH) [15]. Conversely, activation of mesenchymal genes by Snail factors appears to be indirect, at least in part, via downregulation of E-cadherin. Whatever their inductive effects on ZEB1 and ZEB2, Snail factors also regulate epithelial and mesenchymal markers directly [16-18]. Indeed, the emerging consensus in the literature points to Snail1 as the mechanism responsible for initiating EMT in reaction to signal induction [19, 20]. In addition, EMT is linked to a variety of signaling pathways including Transforming Growth Factor-β (TGF-β), Platelet-Derived Growth Factor (PDGF), and Hepatocyte Growth Factor (HGF), Notch, and RAS [21]. A large number of reports represent that cancer cells showed the properties of cancer stem cells during EMT, and that their gene expression profile is approximately similar to cancer stem cells [22]. Mesenchymal stem cells (MSCs) are adult stem cells which can be isolated from post-natal tissue. MSCs are multipotent cells that can divide into three germ layer-lineages, including osteoblasts, adipocytes and chondrocytes, known as the mesodermal lineage [23]. MSCs also have the capacity for proliferation and medicinal properties such as anti-inflammatory and immunomodulatory effects [24, 25]. Therefore, these cells provide a very useful source for cell therapy. Furthermore, paracrine influences are believed to be responsible for the therapeutic results of MSCs that have been shown to suppress not only internal organ fibrosis such as visceral fibrosis but also scaring and fibrosis of body surface tissues such as skin, processes indicated to be regulated by EMT [26-30]. In fact, various research have demonstrated the therapeutic effect of conditioned medium extracted from MSCs on recovery of accidents and autoimmune diseases [31]. Despite the mentioned points, the results of MSC and condition medium of MSC for cancer therapy are controversial. It has been proven that in cancer therapy, MSC has a dual role [32]. Nonetheless, recent research have reported contradictory findings about the stimulatory impact of MSCs on tumor pathogenesis by tumor micro-environmental help and tumor growth stimulation [33]. Mesenchymal stem cells-conditioned media (MSCs- CM) comprises a variety of factors generated and secreted by MSCs, such as Interleukin- 6 (IL- 6), CCl- 5, transforming beta growth factor and cell-derived stromal factor- 1[34]. Researchers have revealed that MSCs-secreted factors including TWIST, MMP, WNT5A, and TGF-β stimulate tumor growth, progression, and metastasis, as opposed to antitumor impacts of MSCs [35]. Condition medium of mesenchymal stem cells from the umbilical cord tends to increase multiform glioblastoma cell proliferation and does not cause apoptosis [36]. The current study was performed as to investigate the effect of BMSC-CM in inducing EMT process in colorectal cancer cells. HT29 cells were

used for confirming whether BMSCs-CM can induce the EMT process.

2. Materials and Methods

2.1 Cell Culture

Colorectal cancer (CRC) cell lines were provided by Pasteur Institute of Iran-Tehran and cultured in RPMI-640, completed with 10% fetal bovine serum (GIBCO) and 100U/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Cells of CRC were incubated at 37°C with atmosphere humidity of 95%, 5% CO2 and 95% O2.

2.2 MSC Isolation and Culture

Human bone marrow mesenchymal stem cells were extracted from iliac crest biopsies. In brief, the bone marrow was extracted in an EDTA tube, and then, the buffy coat layer was isolated through centrifugation (450xg, 10min). The separated buffy-coat with an equivalent amount of Ficoll was centrifuged (400xg, 20min) to obtain MSCs. The harvested cells were grown in a monolayer culture and preserved in a full medium of Dulbecco Enhanced Eagle Medium (DMEM, Gibco-Invitrogen, USA), containing 15% Fetal Bovine Serum (FBS, Gibco-Invitrogen, USA) and 1% Penicillin / Streptomycin (Gibco-Invitrogen, USA) at 37°C and 5% CO2 in a humidified atmosphere. For the purpose of the present study, the bone marrow mesenchymal stem cells were used at passages 4-6. These cells were shown to be positive for flow-cytometry analysis of CD90, CD73 and negative CD34, and CD45 (all eBioscience, USA), and were able of form osteoplastic and adipogenic differentiation based on protocols developed (data haven't seen). Cell morphology modification was studied using contrast-phase microscopy (Olympus IX7071)[37].

2.3 Conditioned Media Preparation

BMSCs were washed twice with phosphate buffered saline (PBS) after 24 h of adherence to the flasks by the 3rd generation of BMSCs. The cells were then cultured with serum-free DMEM, and the supernatant (cells had achieved an 80 percent confluence) was collected after 48h. The obtained supernatant was then centrifuged to 1,000rpm for 10min. The CM was collected with a sterile filter after the supernatants had been re-centrifuged at 3,000rpm for 5min.

2.4 BMSC-CM Treatment

After cells had reached to the confluence of about 80% in the T-25 flask, they are grown at a seeding density of 2x104/cm in two T-25 flasks. The seeded cells were fed through condition medium-MSC FBS free for 24 hours for the purpose of inducing EMT. Next, they replaced the medium with 3ml MSC-condition medium treated for 48 hours. After inducing EMT, cell alteration of morphology was studied with the use of contrast phase microscopy (Olympus IX7071).

2.5 RNA Extraction and Real-Time Reverse Transcriptase-PCR

AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) was used as to extract RNA from CRC cell lines. The concentration and purification of extracted RNA were determined by using Nanodrop spectrophotometer (NanoDrop Techniques INC, USA). The reverse transcribed was used on 50ng of RNA. The Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used for that matter. The cDNA was provided for measuring the expression level of EMT biomarkers of ZEB1,E-cadherin, Vimentin, β-Catenin and Snail genes. GAPDH was used as an internal control in EMT array reaction. The Real-time PCR (PCR) was done in triplicate with SYBR Green Master Mix (Ampliqon, Denmark) on the Rotor-Gene 5plex HRM (QIAGEN, Hilden, Germany). Primers used in this study are shown in Table 1.

Table 1. Sequence of the primers used in the real-time PCR experiment

Gene names	Forward	Revers
E-cadherin	TGCTCTTGCTGTTTCTTCGG	CTTCTCCGCCTCCTTCTTC
β-catenin	GGGTAGGGTAAATCAGTAAGAGGT	GCATCGTATCACAGCAGGTT
Vimentin	CCAGGCAAAGCAGGAGTC	CGAAGGTGACGAGCCATT
Snail	CACTATGCCGCGCTCTTTC	TGCTGGAAGGTAAACTCTGGAT
ZEB1	CAAGAGGCGCAAACAAGC	GGTTGGCAATACCGTCATCC

The expressions of the above genes were normalized to the housekeeping gene. The results were compared using the 2- ΔΔCT formula.

2.6 Statistical Analysis

All analysis was done by the Excel and Graph Pad prism software. Each experiment was applied in three consecutive days and in duplicate. The results were determined as mean±SEM. Student's T-test was used for analyzing differences between the two groups and one-way analysis of variance (ANOVA) was applied for analyzing more than two groups. Finally, the results of the acquired data proved to be statistically significant ($p<0.05$).

3. Results

Alteration of E-Cadherin, β-Catenin and Vimentin as Epithelial-Mesenchymal Transition (EMT) Markers by BMSCs-CM in Colorectal Cancer Cells

The MSC-CM was prepared using human bone marrow isolated MSC. The impact of BMSCs-CM on human colorectal cancer HT29 cell line was tested. The majority of cell treatments with BMSCs-CM gained a fibroblast spindle shape as shown in Fig.1. HT29 cell treatment with BMSCs-CM induced an EMT. E-cadherin, β-catenin and Vimentin are some of the markers related to EMT which have been tested in this study. After BMSC-CM treated HT29, there were some changes in E-cadherin, β-catenin, and Vimentin. E-cadherin is tested as a precursor of epithelial cells. On the other hand, modifications of β-catenin and Vimentin are being investigated as receptors for mesenchyme cells. Real-time PCR tests were used to determine their level of expression. This study manidested that relative to the control group, the level of expression in the epithelial cell marker, E-cadherin, decreased significantly ($p=0.05$). In comparison, mesenchyme cell markers, β-catenin and Vimentin showed substantial increase (β-catenin:0.01; vimentin:0.01) relative to the control group, indicating the transformation of epithelial cells into mesenchymal cells (EMT process), as it is evident from Fig.2.

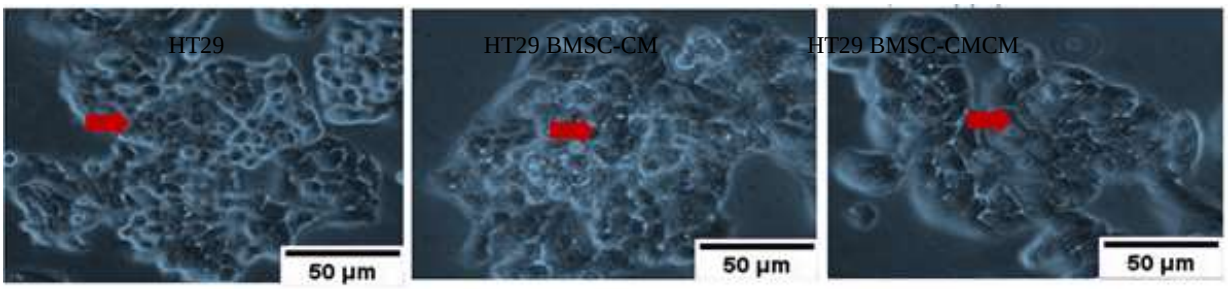

Figure 1. Changes in HT29 cells after treated with 48h and 72h BMSCs-CM observed with an invert microscope magnification of 200x

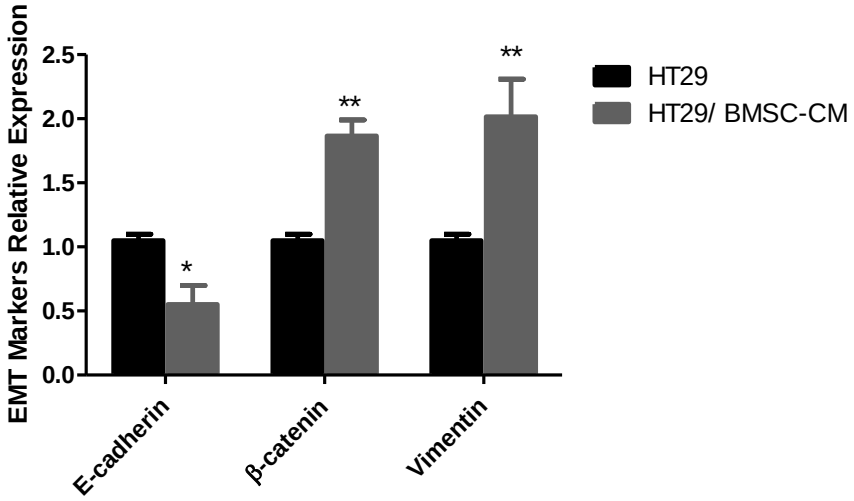

Figure 2. Real-time PCR analyzed the expression of E-cadherin, β-catenin, vimentin in colorectal cell line (HT29) with treated BMSC-CM compared to the HT29 without any treatment group. The expression of EMT markers were normalized by GAPDH as a housekeeping gene. Bar are indicting the MEAN +_SEM of three times experiments. Data represent significant decrease of E-cadherin (P=0.05) and increase of β-catenin (P=0.01), vimentin (P=0.01) after treated with BMSC-CM

Increasing Transcription Factors of ZEB1 and Snail Associated with EMT Markers

EMT is characterized by downregulation of main epithelial markers (the most important being E-Cadherin) and upregulation of mesenchymal markers in order to show the influence of BMSCs-CM on the EMT process in the HT29 cell line. In this work, Real time-PCR analysis was used to test the degree of expression of ZEB1 and snail with EMT markers. Results revealed that the upregulation of ZEB1 and snail resulted in decreased expression of E-Cadherin and increased expression of Vimentin and β-catenin in HT29 cell line. In other terms, after inducing EMT, ZEB1 and snail (ZEB1:P=0.01; Snail:P=0.001) increased dramatically, as seen in Fig. 3.

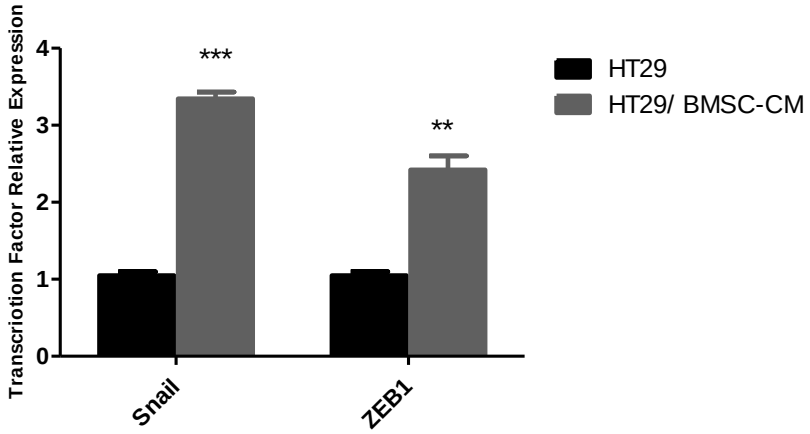

Figure 3. ZEB1 and Snail expression levels in colorectal cell line analyzed with Real-time PCR. Both of these transcription factors enhanced after treated with BMSC-CM Snail (P=0.001), ZEB1(P= 0.01) the expression of transcription factors were normalaized by GAPDH as a housekeeping gene. Bars are indicting the MEAN +_SEM of three times experiments.

4. Discussion

Colorectal cancer (CRC) is the third most prevalent cancer and the second leading cause of cancer-related deaths. Metastasis poses a medical threat to treating colorectal cancer [1]. In other words,

advanced metastasis CRC has no effective therapy [6]. In summary, it is necessary to identify new biomarkers for the diagnosis of CRC and to develop new methods of treatment [7].

Various studies have demonstrated a close association between metastasis and the epithelial-mesenchymal transformation (EMT) phenomenon[9]. Phenotype was acquired by reducing epithelial marker expressions such as E-cadherin and mesenchymal (like Vimentin) [14]. Our assumption of a BMSC-CM has effect on inducing EMT in colorectal cancer. The results manifested that BMSC-CM induced EMT. It was also displayed that BMSC-CM contributed to morphological modification in cell line HT29; that is, to spindle-like cells, it alters the circular cells. Untreated HT29 cells in particular, had a clear epithelial morphology whereas HT29 treated with BMSC-CM showed a mesenchymal shape, which is one of the characteristics of the EMT process. ZEB and Snail transcription factors regulate the EMT process, and the increase in the expression of these two factors results in a shift in the expression of the genes involved in the EMT process. The epithelial cell exhibits mesenchymal stem cell properties through lowering the E-cadherin level and increasing the amount of β-catenin and Vimentin. On the other hand, the molecular-level studies indicated that the characteristics of stem cells in these cells are accompanied by β-catenin and increased Vimentin after BMSC-CM treatment and decreased E-cadherin after BMSC-CM treatment. EMT process relates to zinc-finger factors such as Snail1, Snail2 (Slug), ZEB1/2 [15].

Alternatively, activation by Snail factors of mesenchymal genes tends to be indirect, at least in part, by downregulation of E-cadherin. On the other hand, ZEB factors include many separate domains for interaction with other transcriptional regulators reviewed previously [38]. ZEB1 and ZEB2 cause an EMT through suppression of epithelial markers and mesenchymal activation [39-43] and were evaluated [44, 45]. The present study also suggests a significant increase in the level of the snail and ZEB1 transcription factors in the colorectal cancer cell line (HT29). This study shows that ZEB1 / Snail transcription factor activates an EMT through suppression of the epithelial marker and mesenchymal activation. Activation of mesenchymal genes by Snail influences seems indirect, at least partly, by downregulation of E-cadherin. With their inductive impact on ZEB1 and ZEB2, epithelial and mesenchymal markers are strictly controlled by Snail influences.

On the other hand, Mesenchymal stem cells (MSCs) are adult stem cells which are readily isolated from postnatal tissues.

MSCs also have replication capacities and therapeutic properties such as anti-inflammatory and immunomodulatory activity [24, 25].

Numerous studies demonstrated that MSCs have inhibitory effects on tumor development by inhibiting angiogenesis, they suppress the immune responses [46-49].

Despite all these, the results of MSC for cancer therapy are controversial. It has been proven that in cancer therapy, MSC has a double-edge role [32].

BM-MSCs were also found in another study to trigger the proliferation, migration and in vitro invasion of the prostate cell line PC3 [50].

Mesenchymal stem cells - conditioned media (MSCs- CM) comprise a multitude of elements generated and secreted by MSCs, such as Interleukin- 6 (IL- 6), CCl- 5, transforming the beta growth factor and the stromal factor- 1, derived from cells [34].

Chuan et al. reported that melanoma cells induce EMT after being treated by MSC conditioned medium, and promote the migration and invasion which probably is associated with the TGF-β/Snail signaling pathway. And this was confirmed in a lung

metastasis mice model in vivo [51].
Isabele C, et al. demonstrate that Mesenchymal stem cell-conditioned medium induce Epithelial-to-Mesenchymal-Like Transition in glioma cells [52].
There is a report from Xiaoyin, et al. that human umbilical cord MSC-CM plays a dual role in A549 lung cancer cells. This causes epithelial mesenchymal transition (EMT) and also induce invasion and migration of mentioned cells. MSC-CM promote cell apoptosis together with inhibition of cell proliferation [53].
Results showed HT29 cells associate with BMSC-CM. Also, results revealed that BMSC-CM has EMT-inducing ability which is probably related to paracrine secretion. This also plays a significant role in metastasis in the colorectal cancer cell line (HT29). The EMT model on colorectal cancer was typically caused by BMSC-CM. Real-time PCR examined cells in two experimental groups (HT29 cell with no treatment, HT29 cell with BMSC-CM treatment). Results demonstrated that EMT markers of β-catenin and Vimentin expressions were increased compared with HT29 cells with no treatment. However, E-cadherin expression was decreased in HT29 cell with no treatment. This research revealed that ZEB1 and Snail transcription factors are significant regulators of EMT through epithelial marker suppression and mesenchymal activation, and also establish an aggressive and metastatic condition in colorectal cancer.

5. Conclusion

This model is used in order to understand the mechanism of cancer metastasis. Results pointed to morphological changes which derive from BMSC-CM, and could activate EMT induction. Furthermore, EMT markers of E-cadherina, Vimentin, and β-Catenin are influenced by this process. This confirms that treatment of the cells with BMSCs-CM induces EMT by the overexpression of ZEB1and Snail genes. It indicates that the condition medium of BMSC induces EMT in the tumor micro-environment where it seems to be an interaction between EMT and paracrine secretion .Consequently, this study may also be utilized for drug screening procedures.

Acknowledgement

This article was part of a Ph.D thesis supported by Shahid Beheshti University of Medical Sciences.

Conflict of interest

The authors declare no conflict of interest.

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