RESEARCH ARTICLE

Comparison of Expression Signature of Histone Deacetylases (HDACs) in Mesenchymal Stem Cells from Multiple Myeloma and Normal Donors

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Abstract

Background: Histone acetylation in chromatin structures plays a key role in regulation of gene transcription and is strictly controlled by histone acetyltransferase (HAT) and deacetylase (HDAC) activities. HDAC deregulation has been reported in several cancers. Materials and Methods: The expression of 10 HDACs (including HDAC class I and II) was studied by quantitative reverse transcription-PCR (qRT-PCR) in a cohort of mesenchymal stem cells (MM-MSCs) from 10 multiple myeloma patients with a median age 60y. The results were compared with those obtained for normal donors. Then, a coculture system was performed between MM-MSCs and u266 cell line, in the presence or absence of sodium butyrate (NaBT), to understand the effects of HDAC inhibitors (HDACi) in MM-MSCs on multiple myeloma cases. Also, the interleukin-6 (IL-6) and vascular endothelial growth factor (VEGFA) gene expression level and apoptotic effects were investigated in MM-MSCs patients and control group following NaBT treatment. Results: The results indicated that upregulated (HDACs) and downregulated (IL6 and VEGFA) genes were differentially expressed in the MM-MSCs derived from patients with multiple myeloma and ND-MSCs from normal donors. Comparison of the MM-MSCs and ND-MSCs also showed distinct HDACs expression patterns. For the first time to our knowledge, a significant increase of apoptosis was observed in coculture with MM-MSCs treated with NaBT. Conclusions: The obtained findings elucidate a complex set of actions in MSCs in response to HDAC inhibitors, which may be responsible for anticancer effects. Also, the data support the idea that MSCs are new therapeutic targets as a potential effective strategy for MM.

Keywords: Multiple myeloma - histone deacetylase - mesenchymal stem cell - interleukin-6

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Introduction

Multiple myeloma (MM) is a hematological plasma cell malignancy. Malignant plasma cells mostly exist in the bone marrow (BM) where many interactions between MM cells and the BM compartments such as mesenchymal stem cells (MSCs) promote survival and tumor cell proliferation of MM cells and facilitate angiogenesis, bone destruction, drug resistance, and immune escape as well (Lemaire et al., 2011). Around 80% of patients at the time of diagnosis of MM show bone disease lesion in radiography (Kyle et al., 2003).

Gene expression profiling studies of the bone compartment have been performed in order to determine transcriptional profiles of bone microenvironment MSCs in association with multiple myeloma bone indicating that MSC cells have a distinct transcriptional profile related with the occurrence of bone lesions in MM disease (Todoerti et al., 2010).

MSCs are rare cells and active population in the bone marrow being capable of self-renewal, differentiation, tumor, wound healing, and immunomodulation. These cells are isolated from bone marrow, adipose tissue, cord blood, or a variety of other tissues. MSCs have crucial roles in tumor progression, as previously verified (Bergfeld and DeClerck, 2010). Regardless to both tumor supportive and inhibitory effects of MSCs (Goldstein et al., 2010), most myeloma researches show a supportive effect of MSCs on myeloma cells, suggesting that the novel drugs are able to negate these tumor-supporting effects in the bone marrow (Reagan and Ghobrial, 2012).

Epigenetic histone modifications have a key role in chromatin remodeling. Histone deacetylases (HDACs) can remove acetyl residue from histone tails and increase

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histone and DNA interaction resulting in enhancement the chromatin condensation (Workman and Kingston, 1998; Forsberg and Bresnick, 2001). HDAC enzyme can regulate gene expression by altering histone acetylation in both transformed and non-transformed cells (Minucci and Pelicci, 2006). Recently, these enzymes have attracted the attention of researchers because HDAC inhibitors (HDACis) have been considered as one of the potential target to treat the solid cancers and neoplastic disorders including MM (Secrist et al., 2003). In addition, HDAC deregulation has been detected in some disorders including breast and lung cancers (Weichert, 2009) and chronic lymphocytic leukemia (CLL) (Todoerti et al., 2010).

However, the association between clinicopathological characteristics and HDAC expression pattern in many cancer types has not been clearly understood. The HDAC expression is highly variable from one cancer to another. For example, the overexpression of a specific HDAC could be associated with a good prognosis in one cancer while a bad prognosis in another(Weichert, 2009). Thus, an individual and comprehensive study of HDAC profiling for each cancer type is of a great deal of importance.

The mentioned MM-MSCs and ND-MSCs are obtained from patients with multiple myeloma and normal individual, respectively. However, a comprehensive study of HDAC expression in MM-MSCs has not been investigated. Indeed, to our knowledge, no research has been performed on HDAC expression as a biological property in MM-MSCs. MM-MSCs evolution is poorly understood and they are functionally and genetically controversial (Reagan and Ghobrial, 2012). Some studies suggest that MM-MSCs are intrinsically abnormal and even will remain abnormal when removed from the myeloma cell BM microenvirment, while others studies claim that MM-MSCs depend on this microenvironment and are only temporarily altered at their gene expression level in response to multiple myeloma BM microenvironment cells (Reagan and Ghobrial, 2012). Thus , understanding how normal MSCs are converted to MM-MSCs necessitates in vitro study and niche-like models of ND-MSCs and MM-MSCs cocultured with myeloma cells (Reagan and Ghobrial, 2012).

Researchers have assumed that more effective and specific chemotherapeutic strategies are considered using in vitro models containing MSCs from patients with multiple myeloma(Smith et al., 2010). Thus, we preformed our studies on the role of MSC in the BM microenvironment to elaborate the interaction between MM-MSCs with Myeloma cell line such as U266.

The aim of this study is to determine the gene expression profile of the 10 HDAC isoforms class I and II correlated with clinical outcomes and most promising drug targets, using real-time PCR method in mesenchymal stem cells isolated from a clinically well-documented new cases of MM patient and compared to control group. Additionally, NaBT can inhibit proliferation and induce differentiation of various tumor cells. This work investigates the effects of NaBT on the biological MM-MSCs and the possible mechanism such as alteration of IL6 and VEGFA expression. Also, this work considers the interaction between MSCs and multiple myeloma cells (U266) characterizing the U266 coculture with MSCs. In vitro coculture study between MM-MSC and MM line was performed to investigate the induced apoptosis by MM-MSCs treated with HDACi on myeloma line.

Materials and Methods

Patients and samples

The population of this work included 20 cases consisted 10 MM patients having no treatments and 10 healthy subjects, N, who had undergone orthopedic operation.

All subjects were obtained after informed consent in accordance with the local ethical committee. The average age of this population was 60 y (range 39-81).

Primary culture of human MSCs (hMSCs)

ND-MSCs were obtained from the normal donors who had undergone orthopedic operation. MM-MSCs samples were obtained from the iliac crest. For ND-MSCs and MM-MSCs, mononuclear cells were isolated from whole BM using a Ficoll-Hypaque density gradient (Nycomed, Takeda, Brussels, Belgium) and cultured based on a previously described method (De Becker et al., 2007). In hMSCs of MM patients, a negative selection of CD138 column was performed by MACS in order to remove the malignant plasma cells contamination before the initial culture. In this study, hMSCs were used at passages 2–4 prior to use were characterized at passage 1 (Todoerti et al., 2010) by immunophenotyping (CD90+, CD73+, CD166+, CD105+ and CD45-) as shown in Figure 1, (Xu et al., 2013). Human myeloma cell line U266 was purchased from the stem cell biotechnology (the first Iranian private research center) and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum.

Coculture System including U266 cell lines in presence of MM-MSCs

U266 cell lines were cultured alone or with MM-MSCs treated with or without NaBT. Transwell coculture, 0.4 um pore sized transwell (Corning Life Science) were used to separate U266 cells from MM-MSCs in order to prevent contamination hMSCs with MM cell line. MSCs cells were collected after three-day coculture and stained with Annexin V staining kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol.

Viability assays

hMSCs and U266 cells were cultured in a 48-well plate in the culture media with three concentrations of NaBT (1, 2, and 4 mM). After incubation for 24h, 20 μL of MTT solution (5 mg/mL) was added to each well, followed by 4 h of incubation at 37°C. Also, 20 μl MTT solutions (5 mg/mL) was added to each well followed by incubation for 24, 48, and 72 h at 37°C. The cell proliferation was performed using Vybrant® MTT Cell Proliferation Assay Kit (Life technology, USA), according to the manufacturer's protocol. (Zhuang et al., 2008; Xu et al., 2013). We selected the concentration of NaBT that showed maximum reduction in cell viability (Park et al., 2006).

Molecular karyotyping of MSC cells

Molecular karyotyping was investigated using conventional cytogenetic analysis to rule out the presence of potential genetic alterations of MSCs cells. Conventional cytogenetic analysis were performed according to previously described method (Todoerti et al., 2010) Experiments were performed in the cytogenetics laboratory of Sarem Women's Hospital.

RNA extraction, cDNA synthesis, and quantification of HDAC isoforms

First, total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Then, the RNA properties were evaluated using gel electrophoresis and nonodrop. cDNA was synthetized using Thermo Scientific versoTM cDNA synthesis kit (Thermo Scientific, Ulm, Germany) according to manufacture instruction.

Quantitative real-time PCR

The HDAC, IL-6, and VEGFA expression profiles were quantified using the SYBR® Green PCR Master Mix (Life Technologies) through a StepOne™ Real-Time PCR System. The transcript levels of mentioned three expression profiles were normalized using housekeeping gene, HPRT, and analyzed using the relative quantification 2-ΔΔCt method. Primer sequences are reported in Table 1.

Results

Effect of Sodium butyrate on in vitro cell proliferation of hMSC and U266

The obtained results indicate that the NaBT reduces the cell growth in a dose-dependent manner in both U266 and MM-MSCs. The maximum inhibitory rate of NaBT as high as 59.51% was observed when the mentioned cells were treated with 4.0 mM NaBT for 72 h. Notably, we observed that NaBT affected the viability of hMSCs and U266 with an IC_{50} (the half maximal inhibitory concentration) of 11.21 and 1.7 μM, respectively, as shown in figure 1. The cell proliferation was significantly decreased after the treatment of hMSC and U266 at different concentrations for 72 h (data not shown). A dose-dependent manner of NaBT on cell proliferation of hMSC and U266 was observed.

Effect of expanded MSCs across further passages (from p2 to p4)

First, we performed conventional cytogenetic analysis on MM-MSCs and ND-MSCs isolated to rule out any

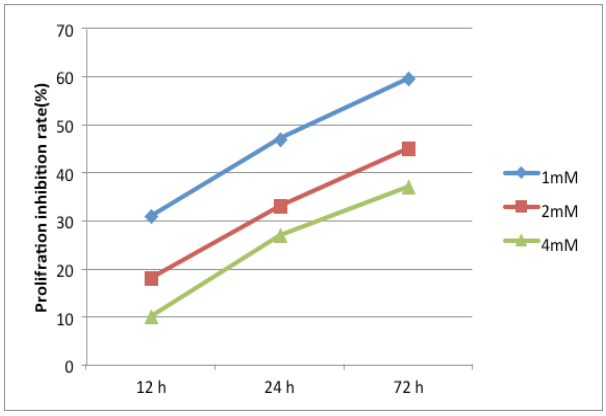


Figure 1. Effect of Sodium Butyrate on hMSC. Anti-proliferative activity of sodium butyrate on hMSCs. IC values were calculated after 72 h treatment

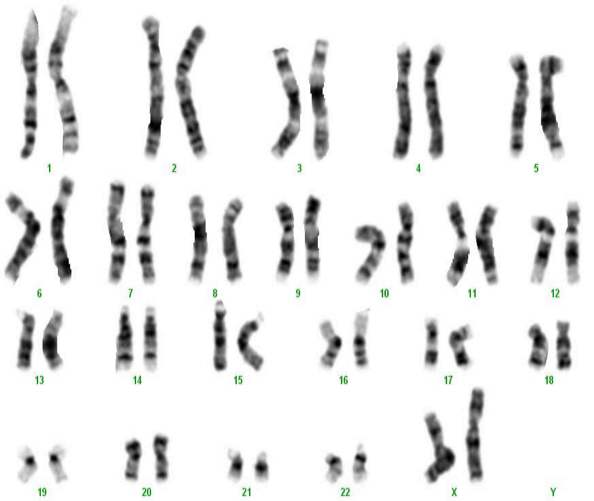
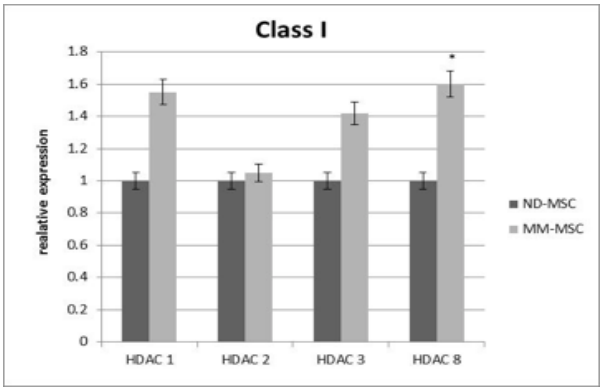


Figure 2. Cytogenetic analysis shows absence of chromosomal abnormality in MM-MSCs



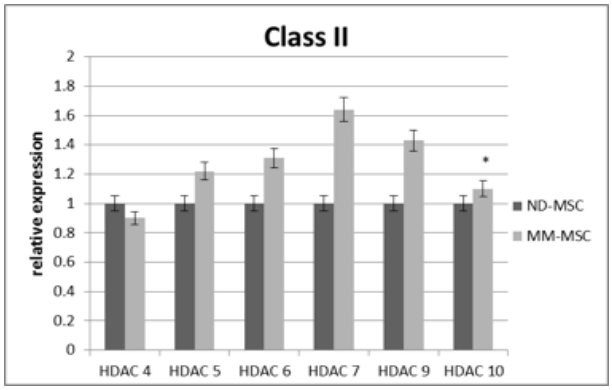


Figure 3. A, B) A and B) Analysis of class I and II HDAC transcript levels by SYBR® Green Real-Time PCR in MM-MSCs vs. ND-MSCs, as described in materials and methods. Samples were run in triplicates and error bars represent standard deviations. A p value <0.05 was considered statistically significant compared to the ND-MSCs

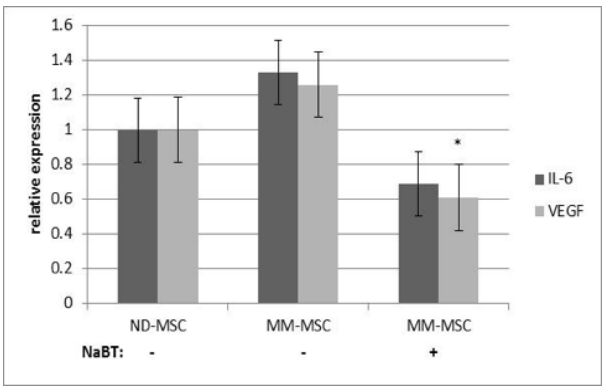


Figure 4. NaBT Suppresses IL-6 and VEGFA growth factor in MM-MSCs treated with NaBT compared with controls. IL-6 and VEGFA transcripts was determined as described in the material and methods. ND-MSCs or MM-MSCs were cultured in medium with (+) or without (-) NaBT. Samples were run in triplicates and error bars represent standard deviations. A p value <0.05 was considered statistically significant compared to the untreated group

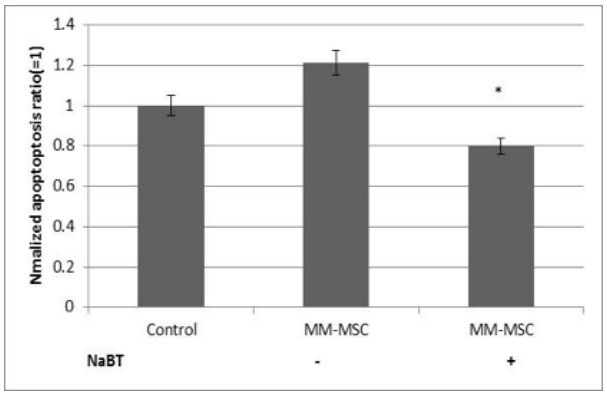


Figure 5. MSCs Treated with NaBT induce apoptosis in U266 line in co-cultured system. Control and MM-MSCs were cultured in medium with (+) or without (-) NaBT. Samples were assay in triplicates and error bars represent standard deviations. A p value <0.05 was considered statistically significant compared to the control group

chromosomal abnormalities. Furthermore, we performed conventional cytogenetic analysis of MSCs at different passages to exclude that MScs expansion could be due to genetic alterations and no chromosomal alterations were observed according to Figure 2. Overall, this work indicates the absence of chromosomal abnormalities.

Gene expression level and qPCR analysis of class I and class II HDAC

qPCR experiments were carried out to assess the relative quantification of HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8 HDAC9, and HDAC10 transcripts in total RNA extracts from ND-MSCs and MM-MSCs. The relative levels of HDAC1-10 transcripts in MM-MSCs cells were compared to those obtained ND-MSCs showing a deregulation of MM-MSCs.

Compared with healthy donor cells, all of the HDACs were significantly upregulated in MM-MSCs samples except HDAC4, which was downregulated, and HDAC2, which was not significantly different. These results showed that HDAC expression was mainly upregulated in MM-MSCs compared with normal ND-MSCs as a control as depicted in Figure 3.

Gene expression level and qPCR analysis of IL-6 and VEGFA

The MM-MSCs was exposed with NaBT to evaluate the effects of HDAC deregulation on MM-MSCs manner and protumoral function. Moreover, qPCR analysis was performed using the primer sets as presented in table 1. The relative levels of IL-6 and VEGFA transcripts in MM-MSCs cells were compared to those obtained from ND-MSCs indicating a significant downregulation of IL-6 and VEGFA in MM-MSCs in presence of NaBT as shown in Figure 4.

The effect of MM-MSC treated and/or untreated with NaBT on apoptosis of U266 cell line

Multiple myeloma cell line was cultured alone or with MM-MSCs. Transwells was used to separate multiple myeloma cells from MM-MSCs. To additionally confirm the anti-proliferative activity of NaBT through downregulation IL-6 and VEGFA, apoptosis assay was performed. The results showed that the NaBT treatment of MM-MSCs coculture results in apoptosis increase of U266 compared to the control, but surprisingly MM-MSCs without NaBT treatment were capable of protecting cells from undergoing apoptosis as shown in Figure 5. The results showed that MM-MSCs is capable of decreasing apoptosis of U266 line compared to the cell line only control (Figure 5). In the case of U266 cells, after normalizing percentage of Annexin V positive cells by dividing values with that of cell line only control, MM-MSCs cocultured group in presence of NaBT had apoptosis ratio of 1.2±0.01, MM-MSCs cocultured group without NaBT 0.8±0.

Discussion

The present work evaluates the HDAC profile in MM-MSC, particularly impact of HDACi on biological properties through IL-6 and VEGFA expression and apoptotic effect of MM line.

There are several evidences showing that HDACs play a key role in different cancers such as in prostate (Halkidou et al., 2004), breast (Zhang et al., 2004) and recruitment of HDACs to suppress growth regulatory gene targets by oncogenic fusion proteins in hematological malignancies (Wang et al., 1998; Lin et al., 2001). Thus, it was assumed that an HDAC deregulation may enhance cancer development/progression (Van Damme et al., 2012). To assessment this hypothesis, we have sought to screen the expression profiles of HDACs (HDAC1-10) of I and II classes due to the sensitivity to the classical HDAC inhibitor in hMSCs and U266 cell line.

Other studies found HDAC inhibitors induce cell cycle arrest at the G0/G1 phase which has been verified in many

investigations (Catley et al., 2003; Mitsiades et al., 2003; Fandy et al., 2005; Kaiser et al., 2006). Moreover, HDAC inhibitors are able to induce apoptosis in myeloma cells. It's noteworthy that its mechanism has not been clearly understood.

Currently, there are no evidences to explore the HDAC expression profile in MM-MSC. Similar to this work, Michael Van Damme et al. have shown that most HDACs in CLL are overexpressed.

The levels of HDAC1, HDAC2, HDAC3, HDAC5 and HDAC6 transcripts in various human and murine cancer cells can be significantly overexpressed by TSA or butyrate treatment has been reported (Gray and Ekström, 1998; Verdel and Khochbin, 1999). Some studies have also suggested to a possible posttranscriptional regulation of HDAC1 expression (Bartl et al., 1997), probably involving the proteasome pathway of targeted protein degradation (Zhou et al., 2000).

In our study, approximately all HDAC were statistically overexpressed in MM-MSC samples compared to control. the overexpression of these HDACs was observed in other cancer types such as breast cancer (Zhang et al., 2004; Ashraf et al., 2006)(17,18) and chronic myeloproliferative neoplasm(Skov et al., 2012) suggesting that HDAC upregulation is often related with cancer development and temporary modified MM-MSC depend on this microenviorment(Van Damme et al., 2012). However, some HDACs (HDAC1, 7, 8, 9) were statistically upregulated in MM-MSCs compared with control samples.

However these alterations in HDAC mRNA expression profiles may have an effect on the biological activities of these cells needs to be further workup.

Our results indicated that the statistically overexpressed HDAC was associated with survival proliferation and apoptosis in MM-MSCs compared with normal group.

IL-6 is a significant growth factor in the proliferation of normal and multiple myeloma plasma cells (Klein and Bataille, 1992; Lauta, 2001; Nishimoto and Kishimoto, 2006). However malignant plasma cells can produce IL-6 through an autocrine signaling feedback, the main source in multiple myeloma patients assumed to be BM MSCs. By comparing the IL-6 and VEGF cytokines gene expression of MM-MSCs versus normal BM MSCs in the presence of NaBT, we observed that NaBT treated MM-MSC exhibit a decrease gene expression level in IL6 and VEGFA. Similar results have been observed for decreased gene expression level of following treatment with HDACi in IL-6 and VEGF (Lu et al., 2008). These results are in agreement with those obtained from coculture system for apoptotic investigation since the IL6 and VEGFA decrease can induce apoptosis in U266 cells.

As the gene expression levels of the IL-6 and VEGFA growth factors are reduced in the presence of NaBT(Smith et al., 2010), we have shown that NaBT could induce apoptosis in U266 in coculture with MM-MSC.

In summary, the effect of HDACs overexpression on disease development has been observed for the first time in the present work. Additionally, it can be concluded that HDAC inhibition enhances the apoptosis induced by NaBT in the MM cell line U266 by inhibiting the IL6 and VEGFA expression. It's noteworthy that the subsequent apoptosis may have important implications to treat MM.

In conclusion, Our finding show that MM-MSCs alone can enhance proliferation and survival of myeloma cells independently while their combination with HDACi diminishes their survival role and only their inhibitory effect on myeloma cell is considerable. In conclusion, results from this study provide evidence in strong support that HDACi can restore MM-MSCs expression pattern (mostly HDAC deregulation) as an important factor in BM microenvironment which leads to decrease supporting effects on U266 cells.

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