Original Article

BIO Treatment Protects Rat Marrow-Derived Mesenchymal Stem Cell Culture Against the TNF-α Induced Apoptosis

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Abstract

Received: 3/Sep/2008, Accepted: 29/Oct/2008

Objective: This study is an attempt to examine the anti apoptotic effects of BIO on rat MSC culture.

Materials and Methods: Rat marrow primary cell culture was established and exposure groups were defined; cultures with 0.01, 0.1, 1 μM BIO. Cells cultured without BIO treatment were used as controls. During culture expantion, the average doubling time, as an index of the rate of cell growth, were determined and compared. To examine whether or not BIO is able to protect MSCs against apoptosis, the passaged-3 cells from each group were induced to undergo apoptosis with the addition of TNF-α (Tumor necrotic factor-α). Three days after, the cultures were quantified in terms of the percentages of apoptotic cells using either the Tunnel or Annexin V staining method.

Results: Marrow cells cultivated with 0.1 and 1 μM BIO appeared to expand at a significantly more rapid rate than the 0.01 μM BIO and the control cultures (p<0.05). Tunnel staining indicated that in 1 μM BIO-treated groups, there were lower percentages of apoptotic nuclei than in groups with other concentrations of BIO (p<0.05). The BIO protective effect appeared to be dose-dependent in that the cultures with high BIO content possessed less apoptotic nuclei. The results obtained by Annexin staining were in agreement with the results of Tunnel staining. The Annexin method additionally takes into account the early apoptotic cells which are not detectable by the Tunnel method.

Conclusion: Taken together, it seems that cultivation with BIO could both increase the growth rate of marrow cells and protect MSCs against induced apoptosis.

Keywords: Mesenchymal Stem Cells (MSCS), Apoptosis, TNF-α, BIO, Osteogenesis, Adipogenesis

Introduction

Mesenchymal stem cells (MSCs) are defined as multipotent cells resident in bone marrow and many other tissues. These cells wre first recognized by Friedenstein et al who provided definitive evidence that bone marrow contained precursors of colonogenic fibroblastic cells in addition to those of well-defined hematopoietic cells (1-2). MSCs are quiescent *in vivo*, starting to enter the cell cycle phase upon *in vitro* cultivation. After several days being in culture, these cells can generate colonies that closely resemble bone and cartilage deposits (1-3). Possessing an extensive self renewal capacity and a multilineage differentiation potential, MSCs were considered to be an appropriate cell resource for cell- and gene-therapy strategies as well as for tissue engineer-

ing approaches (4).The efficiency of the cells in curing the genetic disorder Osteogenesis imperfecta (5), restoration of hematopoiesis after cancer treatment (6), bone regeneration (7-8), cardiac muscle repair after infarction (9) and treating articular cartilage defects (10) have previously been well documents. Moreover, previous research work has revealed that MSCs are able to differentiate into cell lineages other than that of their origin (11). Such ability is referred to as transdifferentiation or plasticity.

In spite of the importance of MSCs for future cell therapy, some aspects of their biology, including cell nature, developmental origin and *in vivo* function remain to be fully understood (12). One limitation associated with the application of MSCs is the reported reduction

in their numbers as a result of cell death occurring following their transplantation into defective tissue. In this regard, the study by Magni et al has indicated that about 70% of MSCs transplanted into the ischemic left ventricle of rats have undergone apoptotic cell death (13). Similarly, Geng et al have reported that about 90% of the cells transplanted into murine left ventricles have been found dead as a result of apoptosis (14). For this reason MSCs need to be protected against apoptotic cell death if transplantation is to be successful.

Apoptosis is a normal physiologic process which occurs during embryologic development as well as tissue homeostasis. This process is regulated by the Wnt signaling pathway that can be activated following the binding of secretary molecules of Wnts on their membrane receptors. This is followed by GSK-3ß inhibition, the consequence of which is inactivation and accumulation of ß-catenin in cytoplasm. Beta-catenin then enters the nucleus and adheres to TCF/LEF that activates the target gene expression (15).

Some research has indicated that BIO (6-bromoindirubin-3–oxim) can act as inhibitor of GSK-3ß by mimicking the action of Wnt secretive molecules (16). Using this property, Sinha et al have reported that the addition of BIO protected a culture of renal epithelial cells against apoptotic changes (17). BIO is one of the derivatives of indirubin obtained from Trypan purple. It adheres to a groove between ATP and GSK-3ß and then inhibits GSK-3ß which results in activation of the Wnt signaling pathway (16).

So far, the effect of BIO on apoptosis has only been reported for renal epithelial cell culture (17). There is no report regarding the effects of BIO on apoptotic cell death in MSCs which are the subject of the present experimental study. The hypothesis is that the addition of BIO to MSC cultures which have been induced to undergo apoptosis will be associated with a decreased number of apoptotic cells. To examine this, rat marrow cells were plated in a medium supplemented with different concentrations of BIO from primary culture to passage 3, at the end of which TNF (a known apoptosis inducer) were added. The cultures were then investigated quantitatively to determine whether or not the presence of BIO is associated with a decrease in apoptotic cell numbers. In addition, the effect of BIO on the rate at which the cells were expanded *in vitro* was determined by calculating their population doubling time during the cultivation period.

Materials and Methods
Marrow cell culture
In the present study, 7 wistar strain rats, 6-8 weeks of age, purchased from the Pastor Institute (Tehran, Iran) were used. The use of animals was approved by the Ethics Committee of the Royan Institute. The animals were sacrificed using excessive chloroform inhalation, the tibia and femur were removed, adherent soft tissue cleaned off and placed into DMEM (Dulbeco's Modi-

fied Eagles Medium, Gibco, Germany) supplemented by 15% FBS (Fetal Bovine Serum, Gibco, Germany) and 100 IU penicillin (Gibco, Germany) and 100 IU streptomycin (Gibco, Germany) in a 15 ml tube. The tubes were transferred into a laminair hood to perform the rest of the procedure: the two ends of the bones were clipped off, a 18 gauge needle was inserted into one end and 2 ml DMEM medium supplemented with 15% FBS and antibiotics was injected into the bone canals to wash out the marrow from the other end into a 15 ml tubes. Bone marrow cells were centrifuged at 1200 rpm for 5 minutes, then re-suspended in 5 ml DMEM containing 15% FBS and antibiotics, counted with a hemocytometer, and finally plated at 10^5-cells/cm^2 in 25-cm^2-flasks. Several flasks of marrow cell primary culture were prepared and three exposure groups defined by supplementation with 0.01, 0.1, 1 µM BIO. Culture without BIO supplementation was used as the control. The cultures were incubated at 37 °C in an atmosphere of 5% CO_2. Two days after culture initiation, the medium was changed and the cultures were continued till confluency, with medium changes of 3 times weekly. Confluent cultures were passaged at 10^5-cells/cm^2 in media supplemented by above-mentioned BIO concentrations. The cultures were expanded through two additional subcultures. In this study passaged-3 cultures were used to conduct the evaluation and experimentation as follows.

Doubling Time (DT)
Doubling time (DT), the time taken for the number of cells to double, is an important index of cell growth rate in culture. In this study, DT was calculated for cultures during passages 1-3 using the equation DT= culture duration/ Population doubling number (PDN). To determine PDN, the cells from each passage were plated at 10^5 cells in 25-cm^2 flasks and allowed to grow for 5 days. At this point the cultures were terminated by tripsinization and the cells lifted and counted with a hemocytometer. Using these data, PDNs were calculated for each passage using the equation PDN= (logN/N0×3.31) where N is the number of cells at the end of culture period and the N0 is the cell number at culture initiation. Total population doubling number (tPDN) was then determined using tPDN=PDN_{p1}+PDN_{p2}+PDN_{p3}. In this study the culture period for determining DT was 15 days (5 days per passage). These calculations were performed for the cells of each of the 7 rats and the data were expressed as average DT values ± standard deviation in hours.

Induction of apoptosis
Passaged-3 cells from BIO-treated groups including the cultures with 0.01, 0.1 and 1 µM BIO and the control were plated in either 25 cm^2-culture flasks or 10-mm cover slips (Sigma, Germany) in a density of 10^5 cells/cm^2. At confluency the medium of each culture was removed and replaced with medium supplemented with

500 U/ml TNF-α (Tomur necrosis factor-α, Sigma, Germany) to induce apoptosis. To determine the protective effects of BIO against induced apoptosis, the cultures were terminated and prepared for further examination 72 hours after culture initiation. The flasks were stained Annexin V to identify the apoptotic cells on the basis of cell membrane associated changes during apoptosis. The cover slips were stained using the Tunnel method to detect the apoptotic cells according to their fragmented DNA.

Tunnel (Terminal Transferase dUTP Nick End Labeling) Staining

Tunnel staining was done using a Tunnel kit (Roche, Germany) according to the manufacturer's instructions. In brief, first, the passaged-3 cells cultivated on cover slips were fixed with 4% paraformadehyde in PBS at room temperature for 1 hour. The cells were then washed with PBS and put in 3% H_2O_2 in methanol for 1 hour at 37 °C followed by a PBS wash. Triton X-100 solution was then applied to the cells at 4 °C for 5 minute, followed by a PBS wash. The cells were then exposed to tunnel solution for 1 hour at 37 °C. This was followed by additional staining with DAB solution for 10 minute at 37 °C in a dark environment. The cells on the cover slips were then dehydrated, cleared, mounted and observed under a light microscope. The apoptotic cells were identified as the cells having dark-stained nuclei. Ten different microscopic fields were observed and all cell nuclei, including dark and normal nuclei, were counted. The data were expressed as average percentages of the dark nuclei ± standard deviation.

Annexin staining and flow cytometric analysis

This staining was performed using an Annexin V-FITC apoptosis detection kit (BD, Germany) according to the manufacturer's instructions. Briefly, the cultures were trypsinized, the cells were lifted, washed with PBS and centrifuged at 500g for 10 minutes. The cells were then transferred into 100 µL staining solution prepared from 10 µL binding Buffer, 10 µL PI, 1 µL Annexin V-FITC, and 79 µL dH$_2$O in a flow cytometric tube and incubated for 15 minutes. After that the cells were suspended in 300 µL binding buffer, centrifuged at 500g for 5 minutes, resuspended in 100 µL binding buffer and transferred to flow cytometry section to determine the percentages of Ann+, PI+, Ann+ PI+ and Ann- PI- cells. This staining was undertaken for the cells isolated from each of the 7 rats and data were expressed as average percentages± standard deviation.

Statistical analysis

ANOVA was used for comparing the values obtained. Tests were performed for the marrow-derived cells of each of the 7 rats. All values stated are means ± standard deviations. A P value of <0.05 is considered to be statistically significant.

Bone and adipose differentiation

Differentiation assays were used to examine the mesenchymal nature of the studied cells.

Osteogenesis

Confluent passaged-3 cells from either the BIO-exposed (experimental) or control groups, plated in 6-well culture plates, were used to induce bone differentiation. For this purpose, the proliferation medium of the cultures was replaced by an osteogenic medium that consisted of DMEM supplemented with 50 mg/ml ascorbic 2-phosphate (Sigma, USA), 10 nM dexamethazone (Sigma, USA) and 10 mM ß-glycerole phosphate (Sigma, USA). The cultures were incubated at 37°C and 5% CO_2 for 21 days with medium replacements three times a week. Occurrence of differentiation was then examined by either alizarin red staining or RT-PCR analysis.

Alizarin red staining

This was used to detect whether or not a mineralized matrix has been deposited among the differentiated cells. For staining, the cultures were first fixed by methanol for 10 minutes, then subjected to alizarin red solution for 2 minutes, washed by distilled water and observed by light microscopy.

Adipogenesis

Confluent passaged-3 cells from all the groups, cultivated in 6-well culture plates, were used to evaluate the adipogenic ability of the studied cells. The proliferation medium of the cells was replaced by adipogenic DMEM medium containing 100 nM dexamethazone (Sigma, USA) and 50 mg/ml indomethasine (Sigma, USA). The cultures were then incubated for 21 days at 37 ºC in 5% CO_2. The medium was changed 3 times a week. Occurrence of adipogenic differentiation was then evaluated by Oil red staining as well as RT-PCR analysis.

Oil red staining

This technique can specifically stain lipid droplets that are produced in adipogenic cultures. To prepare the cells, the culture was fixed with 4% formalin at room temperature, washed with 70% ethanol and then stained with oil red solution in 99% isopropanol for 15 minutes. The dye solution was removed and the cultures were washed with 70% ethanol and observed by light microscopy.

RNA extraction and RT-PCR analysis of gene expression

Total RNA was collected from the cells having been induced to differentiate into osteoblastic and adipocytic lineages as detailed above, using RNX-PlusTM solution (CinnaGen Inc., Tehran, Iran). Before reverse transcription, the RNA samples were digested with DNase I (Fermentas) to remove contaminating genomic DNA. Standard reverse-transcription reaction

was performed with 5 μg total RNA using Oligo (dT) 18 as a primer and RevertAid TM H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Subsequent PCR was as follows: 2.5 μl cDNA, 1X PCR buffer (AMS), 200 μM dNTPs, 0.5 μM of each primer pair and 1 unit/25 μl reaction Taq DNA polymerase (Fermentas). The primers indicated in Table 1 were utilized to detect differentiations. Amplification conditions were as follows: initial denaturation at 94˚C for 5 minutes, followed by 35 cycles of denaturation at 94˚C for 45 minutes; annealing at 72 (ALP), 56 (Osteocalcin), 61 (C/EDP-α), 57(PPAR-Gamma), and 53 (GAPDH) for 45 minutes; extension at 72˚C for 30 minutes; and a final polymerization at 72˚C for 10 minutes. Each PCR was performed in triplicate and under linear conditions. The products were analyzed on 2% agarose gel and visualized using ethidium bromide staining.

Results
Cell culture
Primary cultures of the marrow cells were mainly fibroblastic in their appearance and kept this morphology during the passages.
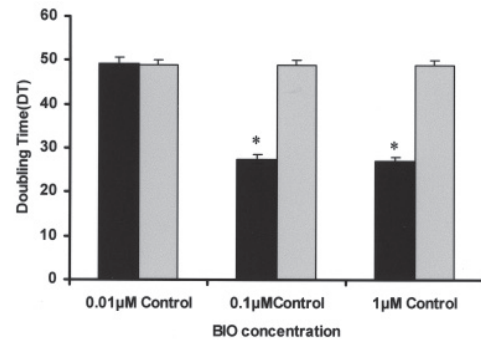

*Fig 1: The graph indicates the DT in the BIO supplemented and control cultures. The growth rate of the marrow cells in cultures with 0.1 and 1 μM BIO appeared to be significantly increased compared with that of cells in the control culture. * indicates significant difference in the BIO groups compared with the control group (p<0.05).*

Doubling time
According to the results, the growth rate of the marrow cells in cultures with 0.1 and 1 μM BIO appeared to be significantly increased compared with that of either control or 0.01 μM BIO culture (p<0.05) as evidenced by their corresponding DT indicated in the graph, Fig 1.
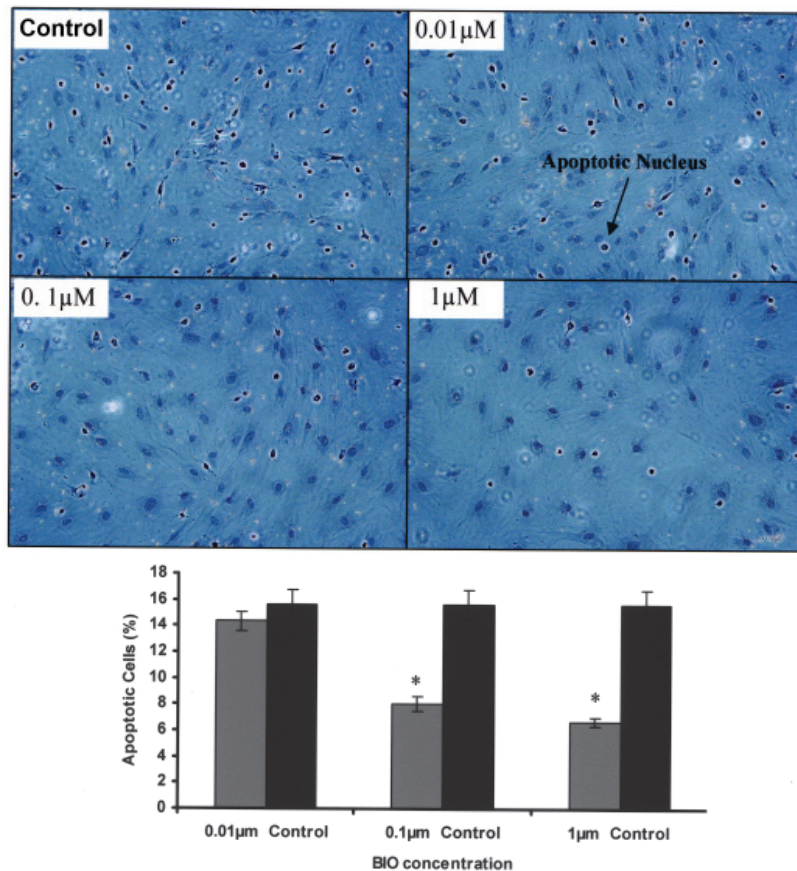

*Fig 2: Tunnel staining. Upper: Microscopic images indicating the apoptotic dark nuclei in the exposure groups. Lower: The graph indicates the percentages of apoptotic nuclei in the control versus the BIO groups. * indicates significant difference in the BIO groups compared with the control group (p<0.05)* **(This figure has also been printed in full-color at the end of the tissue).**
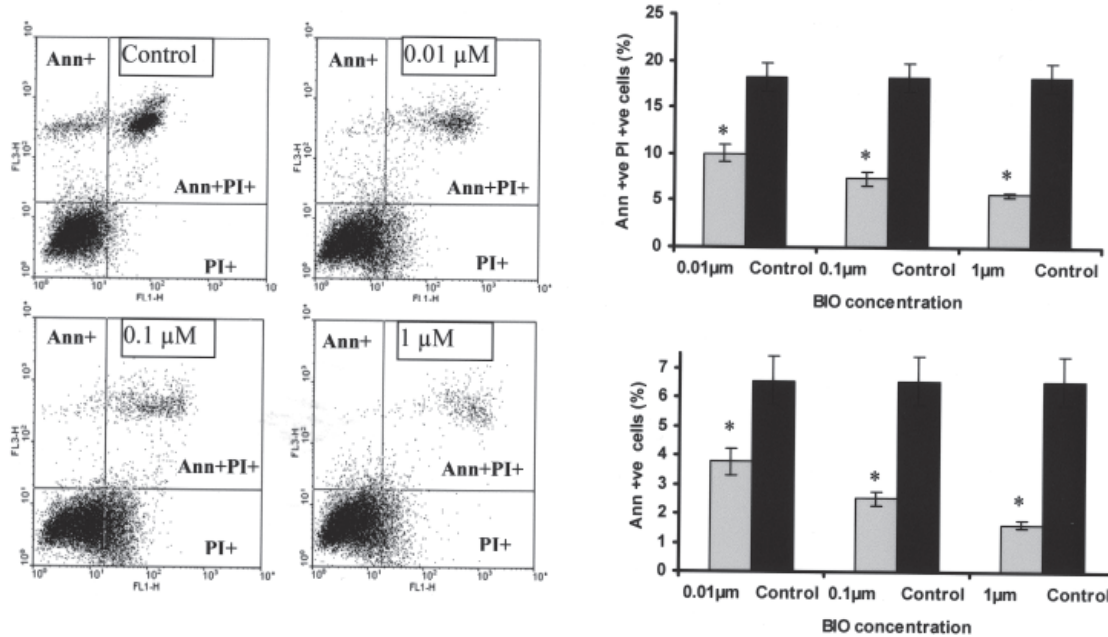
*Fig 3: Annexin V staining and flow cytometric analysis. Upper two rows are a flow cytometric plot indicating the cells as dots in a different zone. The third row is the graph indicating the differences between the BIO-treated and control groups in terms of the percentage of Ann+PI+ (late apoptosis). Lower row is the graph indicating the percentages of the Ann+ cells (early apoptosis) that can not be detected by the tunnel method. * indicates significant difference in the BIO groups compared with the control group (p<0.05).*

In this regard there was no significant difference between the control (DT= 50.575 ± 1.05 hours) and 0.01 µM BIO (DT= 50.97 ± 1.304 hours) cultures and between the 0.1(DT=27.414 ± 1.275 hours) and 1 µM (DT= 26.062 ± 1.00 hours) BIO cultures. These DT values indicate that marrow cells in cultures with 0.1 and 1 µM BIO could reach confluence more rapidly than in cultures with 0.01 µM BIO and the control. Our observations were in agreement with these findings in that we observed that the cultures with 0.1 and 1 µM BIO became confluent in about 7 days, while those with 0.01 µM BIO and the control reached confluence in about 13-14 days.

Tunnel staining

Findings from the tunnel staining, Fig 2, were in agreement with those from the Annexin V staining in that there was a smaller quantity of apoptotic cells in the BIO-treated groups than the control group. Similarly, a dose-response relationship between BIO concentration and apoptosis was obvious. Comparison of the cultures with each other revealed that all the differences are statistically significant (p<0.05) except that between 1(6/7% ± 0.320%) and 0.1 (8% ± 0.57%) µM BIO and that between 0.01 µM BIO (14.34% ± 1/240%) and the control (15.6% ± 1.14%).

Table 1: The Primers that were used For RT-PCR analysis

Genes	Primers	Annealing Temperature (°C)	Product Size (bp)
ALP	F: CGGACCCTGCCTTACCAACTCATTTGTGC R:CGCACGCGATGCAACACCACTCAGG	72	396
Osteocalcin	F: GATTATAGTGACACAGAC R:AGCAGGAATACTAACTGC	56	287
GAPDH	`F: TGCTGAGTATGTCGTGGAGTC R:AAAGGTGGAAGAATGGGAG	53	612
C/EDP-α	F: ACGTGGAGACGCAGCAGAA R: AGGCGGTCATTGTCACTGG	61	340
PPAR-Gamma	F: GGTGAAACTCTGGGGAGATCC R: TGAGGGAGTTTGAAGACTCTTC	57	400

Table 2: The percentages of Ann+, Ann+PI+, PI+ and Ann-PI- cells.

	Control	0.01 μM	0.1 μM	1 μM
An+	6.550 ± 0.84	3.76 ± 0.436	2.52 ± 0.231	1.64 ± 0.12
PI+ An+	18.192 ± 1.533	10.02 ± 0.976	7.358 ± 0.723	5.692 ± 0.206
PI- An-	64.83 ± 3.30	74.90 ± 4.94	77.72 ± 3.21	81.61 ± 3.42
PI+	10.50 ± 4.37	11.40 ± 3.99	12.50 ± 4.02	11.10 ± 2.13

Annexin V staining and flow cytometry analysis

The results of this evaluation are indicated in Table 2 and Fig. 3 as the percentages of An+ (early apoptosis as phosphatydyl serine phospholipids translocation has occurred), PI+ An+ (late stage apoptosis in which the cells are already dead, as phospholipids translocation has occurred followed by loss of plasma membrane integrity), PI- An- (intact cells) and PI+ (necrotic cells) in each culture group. According to these data, all BIO-treated groups appeared to contain a significantly lower percentage of apoptotic cells compared to the control (p<0.05). Again the BIO effect seemed to be dose-dependent in that the highest concentration was associated with smallest percentage of apoptotic cells.
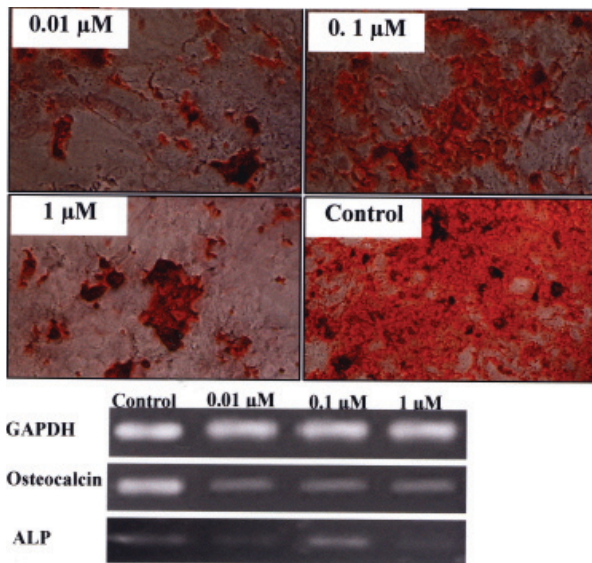

Fig 4: Alizarin red staining and RT-PCR analysis of the osteogenic cultures of cells cultivated in a medium with or without BIO supplementation.

Differentiation

In Fig 4 the deposition of mineralized matrix among the cells can be seen stained red with alizarin red. Red areas in Figure 5 indicate lipid droplets produced by adipose differentiation and stained with Oil red stain. RT-PCR analysis indicated the expression of the osteocalcin and alkaline phosphatase (ALP) genes in all osteogenic cultures (Fig 4) and PPAR-alpha and C/EDP-α in all adipogenic cultures (Fig 5). All these data indicate the MSC nature of the studied cells.
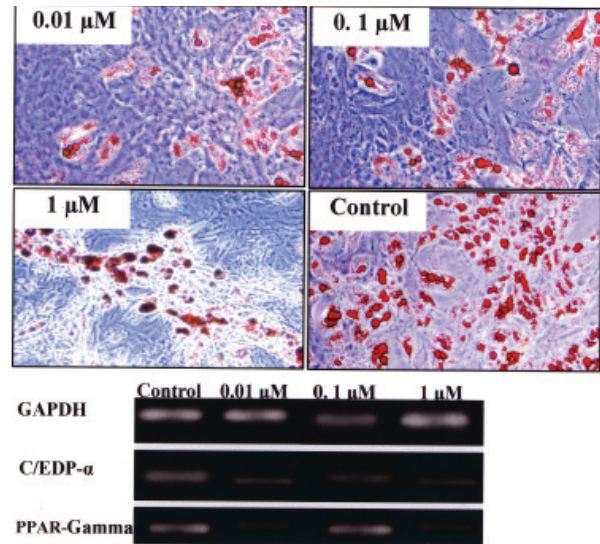
Fig 5: Oil red staining and RT-PCR analysis of the adipogenic cultures of cells cultivated in a medium with or without BIO supplementation.

Discussion

In the present study investigated the protective effect of BIO against experimentally induced apoptosis in rat marrow-derived MSC cultures. Our data indicated that the presence of BIO in the culture medium can result in significant decreases in the proportion of apoptotic cells compared with controls (without BIO treatment). To date, the anti apoptotic effects of BIO have been examined only in renal epithelial cells. (17) Thus, our report on MSC culture is the first investigation in this field. In addition, measurement of cell doubling time during the expansion period revealed that cells in the presence of BIO grew faster than those without. This effect of BIO has already been reported in the culture of hipocampal neurons (18), renal epithelial cells (17) and murine and human embryonic stem cells (19) but not marrow-derived MSCs. All these BIO effects appeared to be dose-dependent in that higher concentrations were associated with higher anti-apoptotic and mitogenic effects. The anti apoptotic effect of BIO could be of great clinical importance since some researchers have reported that the majority of MSCs transplanted into the cardiac wall have undergone apoptotic changes and died (16-14). To solve this problem, one strategy would be to add BIO to cell materials intended for transplant. However, this issue needs more investigation.

Sinha et al in 2005 indicated that the addition of BIO with concentrations of less than 1 μM into renal proximal epithelial cell cultures can protect them against apoptosis induced by growth factor deprivation (17). This work also showed that the anti-apoptotic effects of BIO were dose-dependent. The results of the present study are in agreement with the findings of Sinha et al in that the BIO with a concentration of less than 1 μM displayed dose-dependent anti-apoptotic effects in rat MSC cultures. The study by Sinha et al provided evidences that the anti-apoptotic effect of BIO is the result of activation of the Wnt signaling pathway. In the present study, the Wnt signaling pathway was not studied, rather the consequence of Wnt signaling activation, i.e. apoptosis, was investigated. Whether or not this effect of BIO in rMSCs is the results of Wnt activation requires futher examination.

In apoptotic cells there certain morphological features are associated with the cell membrane and nucleus. A cell membrane-related change is the translocation of the phosphatydyl serine phospholipids from the inner half to the outer half of the plasma lemma resulting in loss of membrane asymmetry. This is an early sign of apoptotic cell death. In the nucleus, the characteristic modification is the internuclesomal cleavage of DNA (20). Tunnel staining can detect the DNA changes but not the early membrane modification. This means that results using tunnel staining are incomplete. In this study, Annexin V staining was also used to capture cells in the early phase of apoptosis (21) which is not detectable using the Tunnel method.

The *in vivo* apoptosis of MSCs used in transplantation have been thought to be due to shortage of the blood supply therein and unknown cellular events (22). In the present study, to expose the cells to apoptosis-inducing conditions TNF-α was added to the cultures. TNF-α is known to be able to initiate apoptotic cell death in cultures by binding to cell surface receptors. Using the TNF-α treatment strategy, Muscari et al in 2005 induced a culture of marrow-derived stromal stem cells to undergo apoptotic cell death (23). Taken together, our results indicate the anti-apoptotic effects of BIO in TNF-α-induced *in vitro* apoptosis. Whether or not this effect is reproducible under *in vivo* conditions requires further investigation.

One important point that needs to be clearly described is that in none of the BIO-treated cultures were the percentages of apoptotic cells reduced to zero. In the other words, only a proportion of the cells died even in BIO-treated cultures. These results thus indicate that even the presence of BIO could not completely protect the cells against apoptotic changes. It seems that in MSC cultures not all cells respond in the same way to TNF-α induced apoptosis and BIO-generated anti-apoptotic protection. In other words, some cells do not respond to BIO treatment and apoptosis continues unchecked. Further investigation is need to clarify the mechanisms underlying this phenomenon.

In spite of considerable efforts to define the antigenic profile of MSCs from animals, no definitive single marker has so far been identified. In almost all studies, the isolation of the cells is mostly performed on the basis of their plastic adherence properties. This means that after isolation, it is necessary to establish their mesenchymal-stem cell properties. In the absence of a specific marker it has been proposed that the gold standard to identify MSCs is their ability to differentiate into two or more cell lineages (24-27), the method of choice in the present investigation.

Conclusion

Taken together our results indicated that BIO is dos-dependently able to protect MSCs against the induced apoptotic changes in cultures. Presence of BIO with either 0.1 or 1 μM concentration causes the cultures to be expanded in significantly more rapid rate than those in control.

Acknowledgments

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