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Isolation and Differentiation of Murine Mesenchymal Stem Cells into Osteoblasts in the Presence and Absence of Dexamethasone

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Abstract.- Bone marrow is rich source of multipotent stem cells that can be easily isolated and differentiated. Mesenchymal stem cells (MSCs) from the bone marrow have multilineage potential and have many therapeutic applications in the form of cellular therapy, tissue repair and regenerative medicine. MSCs differentiation into osteoblasts is a complex process that is regulated by many internal and external factors. We studied the differentiation of MSCs isolated from tibia and femur of mouse into osteoblasts using osteoblast inductive medium in the presence and absence of dexamethasone. Alkaline phosphatase (AlP) activity, total protein concentration, total cell number and mineralization was checked during differentiation process in the presence and absence of Dexamethasone. Total protein of induced and un-induced cells were also analyzed. AlP activity was higher in induced cells as compared to un-induced cells but total protein concentration and cell number was higher in un-induced cells. After differentiation the induced cells were positive for Alizarin Red stain even in the absence of dexamethasone whereas the un-induced cells were negative and very less mineralization was observed. No difference in protein profile was observed in induced and un-induced cells by SDS-PAGE. MSCs were successfully differentiated into osteoblasts even in the absence of dexamethasone. Dexamethasone treatment however accelerates the cell proliferation and induces early differentiation of osteoblasts.

Keywords: Mesenchymal stem cells, osteoblasts, cells, differentiation, Dexsamethasone.

INTRODUCTION

Two types of stem cells reside in adult bone marrow; hematopoietic stem cells and mesenchymal stem cells (MSCs), which have different and distinct lineage potential. Bone marrow stem cells are multipotent in nature and can be easily isolated. They have enormous application in the form of cellular therapy, tissue repair and regenerative medicine (Leblanc, 2006; Phinney and Prockop, 2007). MSCs from bone marrow can be differentiated mainly into adipocyte, chondrocyte and osteoblastic lineages (Pittenger *et al.*, 1999; Owen, 1988; Bianco and Robey, 2000). Differentiation of MSCs into osteoblast is a complex process that is regulated by expression of different transcription factors, mainly Runx and BMP2 and expression of osteoblast specific gene like alkaline phosphatase (AlP) and type 1 collagen. Beside this, change in extracellular matrix and mineralization is also important steps of MSCs differentiation into osteoblasts (Karsenty and Wagner, 2002; Lian *et al*., 1998; Aubin, 1998). Bone homeostasis is maintained by tight collaboration of osteoblasts and osteoclasts and change in homeostasis lead to disease condition (Duplomb *at al*., 2006).

Different studies have shown that environmental, hormonal and local factors play role in differentiation of MSCs into osteoblast (Marie and Fromigue, 2006). Different approaches and methodologies have been utilized to study the molecular mechanism of MSCs differentiation into osteoblasts (Aubin and Triffit, 2002; Ghilzon *et al*., 1999; Toquet *et al*., 1999). In the present study we isolated MSCs from mouse bone marrow and studied the differentiation process using osteoblast induction medium in the presence and absence of dexamethasone.

MATERIALS AND METHODS

Isolation of mice mesenchymal stem cells

Tibias and femurs of 16-week-old Swiss Webster female mice were excised aseptically and cleaned of adhering soft tissues. The epiphysis was cut, and the bone marrow was flushed out using

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DMEM medium (incomplete medium). Single cell suspension was obtained by passing the flushed out cells from 22-gauge needle several times and counted the number of cells by hemocytometer. Complete medium (containing glutamine, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin) was added to cell suspension in 75 cm^2 flasks (Nunc), incubated at 37°C with 5% CO_2 in humidified environment for 24 h. The medium was removed and the cells were washed with PBS to remove all the non-adherent cells. The medium was changed and the flask incubated again. When cells reached confluent stage, the cells were trypsinized with Trypsin-EDTA (PAA).

Osteoblast induction

For osteoblast induction, $2x10^4$ cells were added in each well of 24 well plate (Nunc). Cells were treated with control and osteoblast induction medium (complete medium supplemented with 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate 1 X 10^{-7} M dexamethasone) in duplicate.

Osteblast induction with and without dexamethasone

In another differentiation experiment osteoblasts induction was done with and without dexamethasone (Dex). Briefly, $8x10^4$ cells were added in 6 well plate and treated with control and osteoblast induction medium with and without Dex.

Alkaline phosphatase (AlP) activity

AlP activity was measured in both induced and un-induced cells after 3, 9, 14, 19 and 25 days of culture. For this purpose, the cells were washed twice with PBS and lysed with 100 µl of lysis buffer (0.1% triton x-100). The cell lysate (20 µl) was mixed with 100 µl of Tris-glycine buffer pH 10.3 (50 mM Tris-HCl, 100 mM Glycine, 2 mM $MgCl_2$, 100 µl of p-nitrophenyl phosphate). The reaction mixture was incubated at 37°C for 30 min, reaction was stopped by adding 50 µl of 3M NaOH. All the reactions were done in triplicate and absorbance was measured at 405nm using ELISA reader. ALP activity was measured as U/µl.

Total protein estimation

Total protein quantification of control and differentiating cell was done by Bradford method. For this standard curve was prepared using 5-50 µg/ml bovine serum albumin. The cells were lysed with 100 µl of lysis buffer (0.1% triton X-100). The cell lysate was mixed with Bradford assay reagent and incubated at room temperature for 2 min. Absorbance was measured at 595nm. The cell number was also counted in the control, and the cells with and without dexamethasone treatment.

Alizarin red S staining

Alizarin red S staining was done after growing the cells for 21 days in induction and non-induction media. The medium was carefully aspirated and the cells washed twice with PBS. The cells were fixed with ice cold 70% ethanol for 1 h at 4°C, and then rinsed with dH_2O twice. Alizarin Red S solution was added to cover the cells and incubated at room temperature for 30 min. The wells were washed four times with dH_20 and images were taken by inverted microscope.

Protein analysis

During the differentiation process, both the control and treated cells were lysed directly by lysis buffer (2M urea, 2% SDS, 10 mM DTT, 10% glycerol, 10m M Tris-HCL (pH 6.8), bromophenol blue 1 mg, dH_2O 3ml and 1% PMSF) at day 3, 9, 15 and 19. The samples and heated in boiling water for 4 min, and finally centrifuged at 12000 rpm for 1 min and 5 µl sample was loaded on 12% SDS-PAGE.

RESULTS

Mesenchymal stem cells

Mice MSCs were successfully isolated after incubating the flushed out cells from bone marrow for 24 h under standard incubation conditions. After 24 h the MSCs were attached to the surface of plate and after 4-6 days they appear spindle shaped (fibroblasts like cells) which is typical morphology of MSCs (Fig. 1A). Medium was changed and the cells were grown to confluency (Fig. IB).

Alkaline phosphatase activity

The ALP activity was significantly upregulated in the differentiating cells as compared

to control cells (Fig.2A). There is also an increase in ALP activity of differentiating cells with and without dexamethasone (DWD and DWOD) as compared to control cells. There is however, not much difference in ALP activity of DWD and DWOD cells (Fig. 2B).

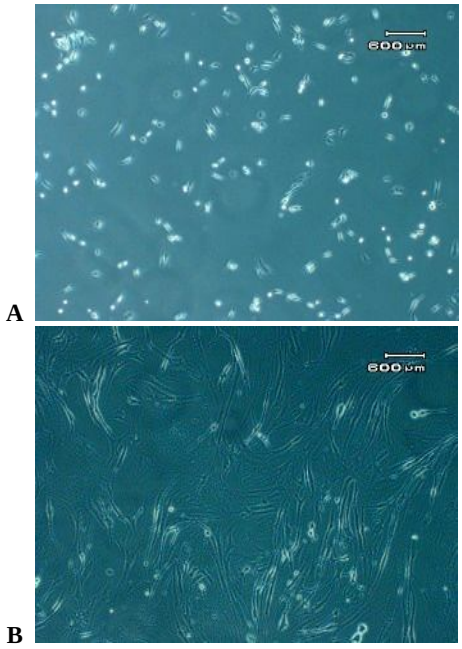

Fig. 1. Murine MSCs after 24 h of growth (A) and at confluency (B). The isolated cells have true spindle shaped morphology of mesenchymal stem cells.

Total protein content

Quantification of total protein was done by Bradford assay and it was observed that the quantity of protein is higher in control cells as compared to induction medium treated cells and on day 9 and 14 there was marked increase in protein quantity (Fig. 3A). Similar results were obtained in the case of other differentiation experiment in which differentiation was done with and without dexamethasone. The total protein quantity was less in DWD cells compared to that of DWOD cells but in control the total protein was much higher (Fig. 3B). Total number of cells were also counted in control, DWD and DWOD cells and there was huge difference in the number of cells in DWD compared to control and DWOD (Fig. 4).

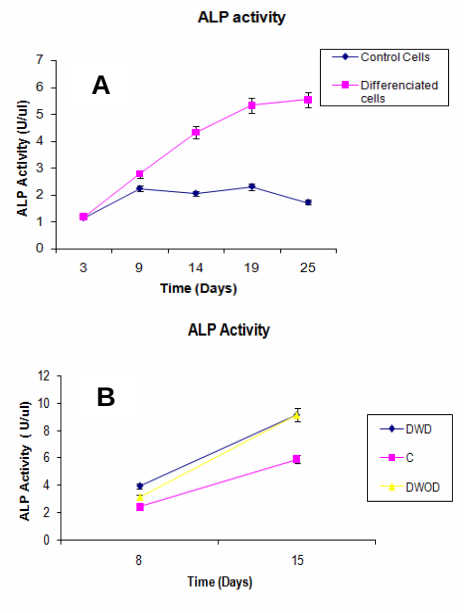

Fig. 2. ALP Activity of control and differentiated cells. at various time intervals (days) (A). ALP activity of control and differentiated cells with (DWD) and without (DWOD) dexamethasone on day 8 and 15 (B).

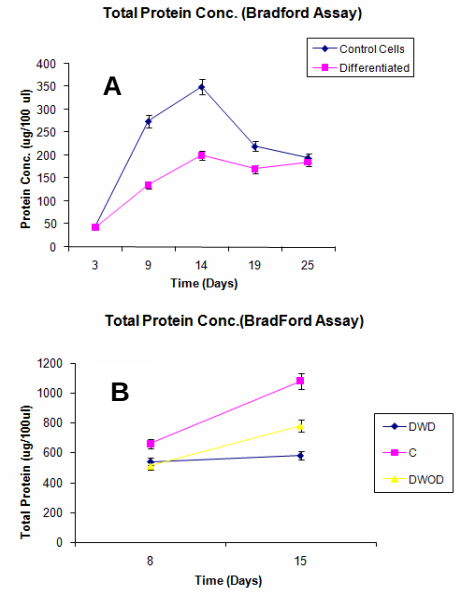

Fig. 3. Total protein content of control and differentiated cells at various time intervals (days) (A). Total protein of control and differentiated cells with (DWD) and without (DWOD) dexamethasone on day 8 and 15 (B).

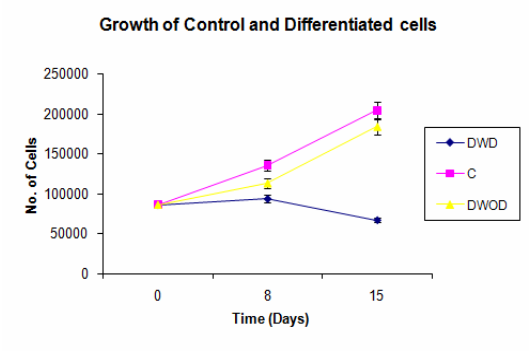

Fig. 4. The cell count of control (C) and differentiating cells with dexamethasone (DWD) and without dexamethasone (DWOD) in day 8 and 15 of differentiation period.

Alizarin red S staining

The control cells and those with osteoblast induction medium were stained with Alizarin red S after 21 days of differentiation period. Alizarin red S stained mineral deposit areas red. Treated cells were positive whereas control cells were negative (Fig.5).

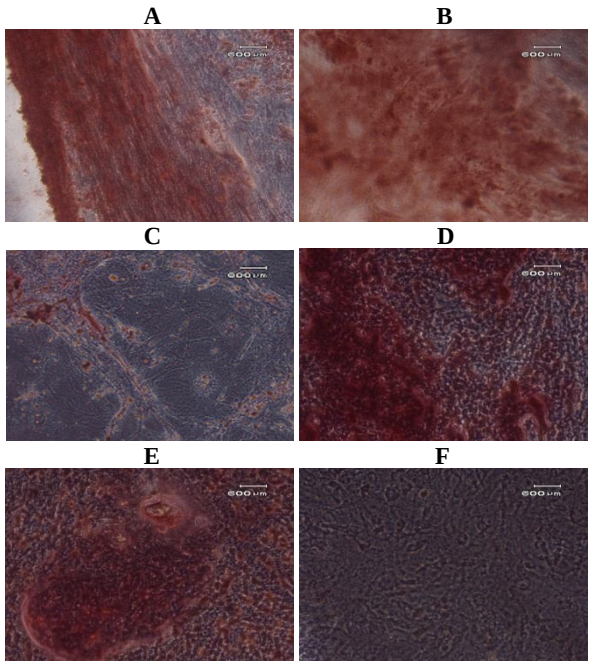

Fig. 5. Alizarin Red S staining of control and differentiated cells. A, 10X image of differentiated cells; B, 40X image of differentiated cells; C, Control; D, DWD; E, DWOD; F, Control cells..

Protein analysis

Total proteins of control and differentiated cells was analyzed on 12% SDS-polyacrylamide gel to determine differences in the expression pattern of proteins. There was no difference in the expression of protein in the two types of cells (Fig. 6).

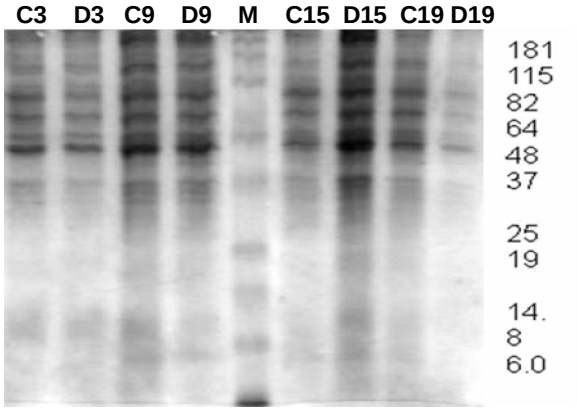

Fig. 6. 12% SDS PAGE pattern of induced and un-induced total cell protein. C is control and D represents differentiating medium, 3, 9, 15 and 19 are the number of days after induction. There is no marked difference in expression of protein in control and differentiating cells and band pattern is almost the same.

DISCUSSION

In the present study the differentiation of MSCs into osteoblasts were studied and markers of differentiation (ALP activity, mineralization) were analyzed. Additionally MSCs were differentiated in the presence and absence of dexamethasone and its effect was analyzed on ALP activity, total protein, cell number and mineralization. Several methods are in use for the isolation of MSCs such as negative and positive selection of cells (Nadri and Soleimani, 2007), cell sorting (Van Vlasselaer *at al*., 1994), applications of cyto-toxic materials (Modderman *et al*., 1994), but all of these methods have different impact on MSCs proliferation and differentiation. In the present report a simple straightforward method was used for the isolation of MSCs. The cells were successfully isolated and they were small and spindle shaped after 24 h incubation in culture medium and after a few more days of culturing they showed the morphology of fibroblasts like cells.

Increase in ALP activity is a marker of MSCs differentiation into osteoblasts as expression of ALP is less in MSCs as compared to mature osteoblasts (Karsenty and Wagner, 2002; Lian *et al*., 1998). ALP expression starts with the induction of differentiation and its level increases as differentiation progresses to osteoblasts (Stein *et al*., 1990; Weinreb *et al*., 1990). As indicated in Fig. 2A the level of ALP was much higher in differentiating cells as compared to that of non-differentiating cells and this difference was maximum on day 25. In another experiment when ALP level was measured in the presence or absence of Dexamethasone, no significant difference was observed, whereas ALP level was high in induced cells as compared with that of un-induced cells. As compared to ALP level when we measured the total protein concentration of induced and control cells, the total protein concentration was much higher in control cells than in induced cells. In an other experiment when we checked the total protein concentration in un-induced and induced cells with and without dexamethasone, the total protein concentration was higher in un-induced culture compared to induced and the level of total protein was higher in induced culture without dexamethasone. The total protein concentration was in consensus with the total number of cells in control and induced culture. The number of cells was maximum in control cells followed by induced cells without dexamethasone and least was in induced cells with dexamethasone (Fig. 4). This is in accordance with previous studies that prove that dexamethasone suppresses the proliferation of the cells (Walsh *et al*., 2001). In the presence of induced medium cell proliferation is reduced but differentiation process is accelerated and cell proliferation is further reduced in the presence of dexamethasone in induction medium.

Extracellular mineral deposition is one of the markers of osteoblast differentiation (Lian *at al*., 1998). Calcium phosphate deposition can be detected by alizarin red staining which is a type of histochemical assay, most frequently used to study osteoblast differentiation. In the present study induced cells were positive for alizarin red staining and un-induced cells did not stain red (Fig. 5). There was significantly higher mineral deposition in induced cells. There is no difference in induced stained cells with and without dexamethasone but un-induced cells totally failed to stain with alizarin red due to lack of mineral deposition. So the staining results indicate that differentiation can be done into osteoblasts even in the absence of dexamethasone but other researchers have shown that dexamethasone promotes the differentiation process (Atmani *at al*., 2002; Cooper *at al*., 1999).

No difference in band pattern of proteins was observed on SDS-PAGE profile of total protein during the differentiation process from induced and un-induced culture. There might be little bit difference in expression of protein especially the transcription factors and that can't be resolved by SDS-PAGE. 2D-Gel Electrophoresis can give a better picture of differences in expression of protein in induced and un-induced culture during the differentiation process.

To conclude MSCs were successfully isolated using a simple procedure from mouse bone marrow and successfully differentiated into osteoblasts like cells as shown by raised ALP activity and mineralization. Dexamethasone is shown to have negative effect on cell proliferation but it stimulates the process of differentiation.

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