

Heat Shock–Induced Enhancement of Osteoblastic Differentiation of hTERT-Immortalized Mesenchymal Stem Cells

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ABSTRACT: Heat shock (HS)–induced stress response in human cells results in a variety of biological effects and is known to induce the transcription of heat-shock proteins, which help the cells to cope with different kinds of stress. We have studied the effects of HS on the differentiation of human mesenchymal stem cells (hMSCs) into osteoblastic cells. As a model for hMSCs we used a telomerase-immortalized hMSC line designated hMSC-TERT. Cells were exposed to 1 h HS at 41°C, 42.5°C, or 44°C prior to incubation in a medium containing either 10^{−8} M 1 α ,25-dihydroxy-vitamin-D₃ (calcitriol) or 10^{−8} M calcitriol, 50 μ g/mL L-ascorbic acid, and 10 mM β -glycerophosphate followed by an analysis of induction of osteoblast differentiation and the formation of mineralized matrix, respectively. Our results indicate that the exposure of cells to mild heat stress enhances the extent of differentiation of hMSCs by 12% to 42%. These effects are an expression of the phenomenon of mild stress-induced hormesis.

KEYWORDS: osteoblast; differentiation; stress; hormesis; human mesenchymal stem cells

INTRODUCTION

Human mesenchymal stem cells (hMSCs) can differentiate into a variety of cell types including osteoblasts, adipocytes, chondrocytes, and endothelial-like cells.¹ In order to improve therapeutic use of stem cells it is important to find

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ways to enhance the qualitative and quantitative extent of differentiation, and thereby increase the chances of the cells to grow into the right kind of tissue or structure. Therefore, we have examined the effects of heat shock (HS) on the differentiation of hMSCs as measured by the synthesis of an osteoblastic marker, alkaline phosphatase (ALP), and cell matrix mineralization. As a model for hMSCs we used a telomerase-immortalized hMSC line designated hMSC-TERT. The results indicate that hyperthermia could be used as a differentiation promoter and an efficient way of making stem cells differentiate to a greater extent.

EXPERIMENTAL METHODS

The hMSC cultures were established from bone marrow aspirates taken from the iliac crest of young donors (men and women between 25 and 30 years old).² The establishment and characterization of the hMSC-TERT cell line have been described previously.³ For cell differentiation assays, cells were seeded in 6- or 96-well plates at a density of 10^4 cells/cm² and incubated at 37°C until 50–60% cell confluence. For osteoblast differentiation studies, cells were grown in medium containing 10^{-8} M calcitriol ($1\alpha,25$ -dihydroxy-vitamin-D₃–Leo, Ballerup, Denmark). For *in vitro* mineralized matrix formation studies, cells were grown in medium containing 10^{-8} M calcitriol, 50 μ g/mL L-ascorbic acid, and 10 mM β -glycerophosphate. The medium was replaced every second or third day. The number of cells in each well was estimated by either MTT assay, crystal violet assay, or by using a standard growth curve. Control cells were counted in triplicate every day from day 0 to day 8 using a Coulter Counter.

For HS studies the cultures were exposed to 41°C, 42.5°C, or 44°C HS for 1 h using a thermostatic water bath controlled to $\pm 0.1^\circ\text{C}$. To avoid any possible loss of water caused by hyperthermia, the medium was removed and substituted after HS. Alkaline phosphatase (ALP) was quantified using a colorimetric assay using *p*-nitrophenol phosphate (PNP) as a substrate.⁴ When exposed to ALP enzyme a yellow reaction product is formed by the dephosphorylation of PNP into *p*-nitrophenol. Cells were washed twice with PBS and incubated with 100 μ L of an alkaline buffer solution and 200 μ L of ALP substrate solution (alkaline buffer solution containing 5 mM PNP) for 30 min at 37°C. The cells were washed with distilled water and specific ALP activity (expressed as nanomoles of PNP/min/ 10^4 cells) was measured at 410 nm and quantified against a standard curve of *p*-nitrophenol.

The mineralized matrix was stained with Alizarin red S and the amount of the mineralized matrix was quantified by modification of previously described methods.^{5,6} Alizarin red S is a dye that binds selectively to calcium salts and is widely used for calcium mineral histochemistry.⁷ Alizarin red S (1 mole) binds 2 mole of calcium in an Alizarin red S-calcium complex. The cells were washed with PBS and fixed in ice-cold 70% ethanol for 30 min followed by

staining with Alizarin red S for another 30 min and washed again. Finally, the stained cells were incubated in 10% (wt/vol) cetylpyridinium chloride to elute bound stain. The dye was collected and absorbance was measured at 570 nm. Alizarin red S in samples was quantified according to a standard curve and expressed as micromoles of bound cetylpyridinium chloride per 10⁴ cells.

RESULTS AND DISCUSSION

Previous studies with hTERT-MSC in our laboratory have shown that the maximum level of HS protein 70 (Hsp70) is reached 3 h post HS (data not shown). Therefore, the cells were treated with HS 3 h prior to adding the medium promoting osteoblast differentiation and mineralized matrix formation, respectively. Three sets of similar experiments were carried out using HS temperatures of 41°C, 42.5°C, or 44°C.

Level of ALP after HS

FIGURE 1 shows that untreated control cell cultures produced a basic amount of ALP after 8 days in culture. Adding calcitriol to the medium showed more than a doubling of the amount of ALP in control cells after incubation for 8 days. However, cells preexposed to hyperthermia had higher levels of

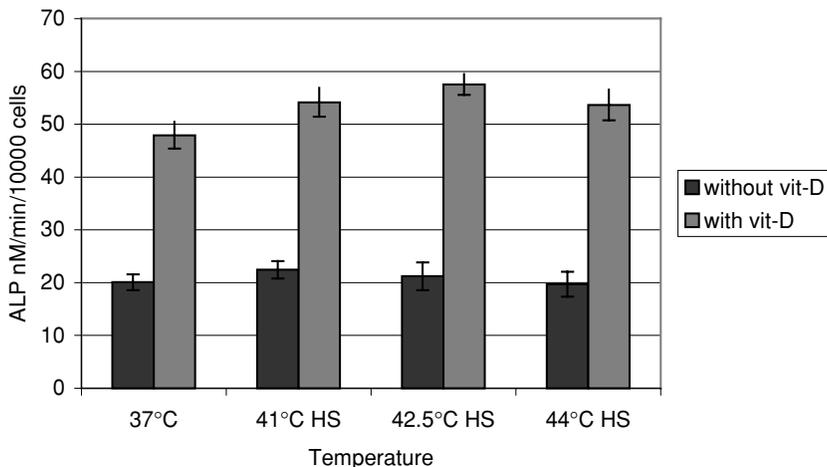


FIGURE 1. Effect of heat shock on calcitriol-induced differentiation of hTERT-MSCs as measured by the levels of alkaline phosphatase (ALP).

calcitriol-induced ALP, which were 12% to 20% higher depending on the temperature. These results suggest that a 1-h HS of 42.5°C is the most beneficial pretreatment to give to hMSC-TERT cells while inducing them to differentiate into osteoblasts.

Amount of Mineralized Matrix Formation After HS

FIGURE 2 shows that untreated control cell cultures had a low level of mineralized matrix formation after 8 days in culture. Adding calcitriol, L-ascorbic acid, and β -glycerophosphate to the medium showed a large increase in the amount of mineralized matrix after incubation for 8 days. As in the case of ALP, the formation of a mineralized matrix was 28% to 42% higher in cells preexposed to hyperthermia. These results suggest that a 1-h HS of 42.5°C is the most beneficial pretreatment to give to hMSC-TERT cells while inducing them to form a mineralized matrix.

Finally, it seems that mild heat stress-induced increase in the levels of Hsps enhances the responsiveness of stem cells to differentiate. These results have practical applications in the therapeutic use of stem cells where a preexposure of cells to mild stress may improve their biological activity and function. Such beneficial effects of mild heat stress on stem cell differentiation are novel examples of the phenomenon of hormesis.⁸ Our studies are in progress to elucidate the exact mechanism of the hormetic effects of mild stress on stem cells and during aging.

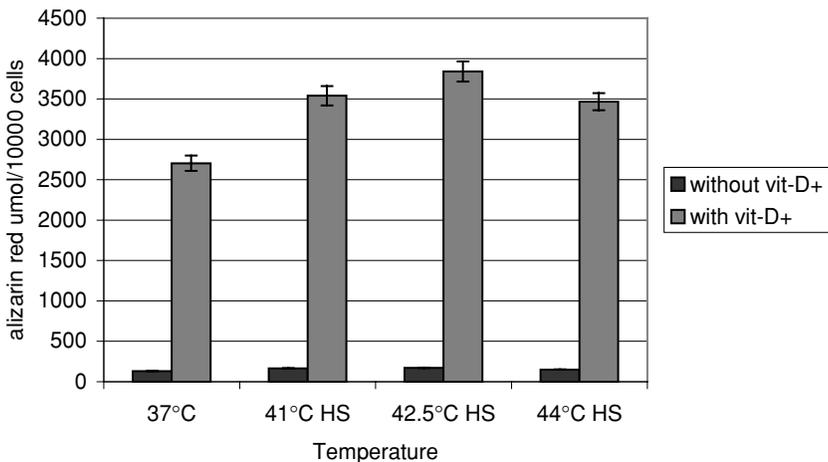


FIGURE 2. Effect of heat shock on the mineralized matrix formation by hTERT-MSCs treated with calcitriol, L-ascorbic acid, and β -glycerophosphate (vit-D+).

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