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Stability of Bone Marrow Stromal Cells to Low Temperatures Depending on Degree of Differentiation

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Abstract—Based on a previous study of the stability of a heterogenous population of keratinocytes against cold depending on their degree of differentiation, we studied in vitro the stability of rat bone marrow stem cells (BMSCs) against cold before and after their differentiation in the adipogenic or osteogenic direction. It was shown that, after the induction of differentiation, BMSCs were least stable against the action of low temperatures than the undifferentiated cells. The obtained data can serve as a basis for the further study of processes and mechanisms that affect the stability of BMSCs against cold depending on their degree of differentiation.

Keywords: BMSC, adipogenic and osteogenic differentiation, low temperature.

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INTRODUCTION

When studying the influence of cold on body organs and tissues, attention was focused on the specific reactions of different types of cells. Authors studying the effect of low temperature on the destruction of cells noticed different optimal cooling rates for different types of cells (McGrath, 1974; Mazur, 1977; Teryman, 1974). In their opinion, these differences may depend on the size of the cell, its surface geometry, its water content, and the permeability of its plasma membranes. Recently, processes of entropy began to be studied, with the suggestion that they can affect the cell stability against the action of cold (Wolf, 2002). This suggestion remains in place, and all of the main approaches to treating diseases using cryosurgery are formed based on classical cryobiology and the methods developed for cell cryoconservation. The particular mechanisms of the stability of body cells and tissues during the cooling process are currently still unclear and require systematic study.

Previously, we studied the resistance to the action of low temperatures of keratinocytes at different degrees of differentiation (Raydan et al., 2011). The obtained results have shown that, in different cooling regimes, stem and transitory cells have higher resistances, whereas under the same conditions, those that are differentiated die. This agrees with the concept of cryosurgeons about the character of the action of low temperature on human skin. Furthermore, the question arose as to whether the revealed regulation is true for other cells at different degrees of differentiation or if it is a characteristic feature of only keratinocytes.

In connection with this, the present work deals with studying the resistance of bone marrow stromal cells (BMSCs) to low temperatures before and after their differentiation in the adipogenic or osteogenic direction. The obtained results indicate that, depending on their degree of differentiation, BMSCs also differ in their degree of stability against the action of cold.

MATERIALS AND METHODS

Isolation of rat BMSCs. Two-month-old outbred rats were used in the work. The animals were anesthetized with ether, sacrificed by stretching the vertebrate column, and sterilized in 70% alcohol for 10–15 min. Femoral bones were taken to isolate bone marrow; they were washed in phosphate buffer (PBS) without calcium and magnesium with the addition of gentamicin (Invitrogen, Great Britain) at a concentration of 50 μg/ml; epiphyses were cut out and the bone marrow was carefully washed out with a syringe with a 23G needle. To isolate the cells containing nuclei, the obtained mixture was suspended in 2 ml PBS, layered upon 5 ml of Histopaque (Sigma, United States) with density of 1.077 g/ml, and centrifuged for 20 min at 800 g at room temperature. The cells present in the Histopaque interphase layer (mainly those containing nuclei) were transferred into another test tube and centrifuged in ten volumes of PBS at 600 g for 15 min at room temperature for purification from Histopaque. The washed-out cells were transferred into plastic Petri dishes (Nunc, Denmark) and cultivated in αMEM medium (Sigma, United States) containing

10% calf embryo serum (HyClone, United States) and a mixture of penicillin and streptomycin (1 : 100 Invitrogen, Great Britain) at 37°C in the atmosphere of 5% CO_2. The cells were counted under an inverted microscope using a Goryaev chamber. The cells were reseeded using a mixture of 0.25% trypsin and 0.02% EDTA in physiological buffer (Invitrogen, Great Britain).

Differentiation in adipocytic direction. To induce of adipogenic differentiation, the described method (Reyes et al., 2010) was used. The BMSC suspension (2×10^4 cells/cm^2) was cultivated in medium containing 90% αMEM medium, 10% horse serum (Sigma, United States), 50 μg/ml gentamicin (Invitrogen, Great Britain), 10^{-9} M dexamethasone (Sigma, United States), 50 μg/ml sodium ascorbate (ICN, United States), ITS solution (100-fold solution, including insulin, transferrin, sodium selenite at concentrations not indicated by the manufacturer) (Invitrogen, Great Britain) and LA-BSA (100-fold solution containing 1 μg/ml linolic acid and 100 μg/ml bovine serum albumin) (Sigma, United States). The induction of differentiation was performed for 2 weeks. The medium was changed every 3–4 days. The degree of cell differentiation was estimated by their lipid content (Reyes et al., 2001). Oil Red solution (Sigma, United States) was prepared immediately prior to cell staining. For this purpose, 0.5% dye in isopropanol was diluted with distilled water in a 3 : 2 ratio and filtrated through the filter with a pore diameter of 0.2 μm. The cells were washed with PBS, fixed with methanol for 2 min at 20°C, washed with 50% ethanol, and stained with Oil Red for 10 min. The stained cells were washed again with 50% ethanol, rinsed with distilled water, and air dried.

Differentiation in osteogenic direction. A suspension of BMSC (1×10^4 cells/cm^2) was transferred into the inducing medium containing 90% αMEM, 10% horse serum (Sigma, United States), 50 μg/ml gentamicin (Invitrogen, Great Britain), 10^{-8} M dexamethasone (Sigma, United States), 50 μg/ml sodium ascorbate (ICN, United States), and 10 mM sodium β-glycerophosphate (Sigma, United States) and cultivated for 2 weeks. The change in the medium was performed every 3–4 days of cultivation.

The degree of cell differentiation was estimated using the reaction for alkaline phosphatase. The cells were washed three times with PBS and fixed with 4% formaldehyde (Sigma, United States) for 1 h at room temperature. The fixed cells were washed three times with PBS, then stained for alkaline phosphatase with a mixture BCIP-NBT (Sigma, United States) for 20–40 min in darkness at room temperature. The stained preparations were washed three to four times with distilled water and dried.

The fraction of the stained cells was determined by photocolorimetric analysis. The cells were washed twice with PBS, fixed in 70% ethanol for 30 min, and added 100 μl of Gencyan violet into each well for 30 min, then washed with water at room temperature for 24 h. The cells were lysed with 10% acetic acid for 15 min and the optical density was measured using a Charity analyzer (Constructor Bureau Probnauchpribor Ltd, Russia).

Exposure to low temperatures. The rat freshly isolated BMSC were cultivated in Petri dishes until the second passage, then differentiation was induced in the adipogenic or osteogenic direction. After this, the cultivated cells were converted into a suspension. Cells in the form of a suspension, as well as in the form of sediment obtained after the centrifugation of the cell suspension, were cooled at different temperatures with liquid nitrogen vapors in 1.8-ml cryoampules (NUNC, Denmark) in the chamber of a programmed freezer (Ice cube 1810, CSYLAB, Austria) with electron block of the control. The rate of cooling to temperatures of −10, −20, and −30°C was 5°C/min and that to temperatures of −40°C and lower was to −12°C/min. The time of the presence of cells at the final temperature always amounted to 5 min. The rate of cooling and the time of exposure were chosen based on our previous study (Raydan et al., 2011).

RESULTS

After 3 weeks of cultivation in inducing medium, the maximal fraction of BMSCs differentiated in the adipogenic (Figs. 1a, 1b) or osteogenic (Figs. 2c, 2d) direction was achieved; therefore, in experiments, cells were taken after this time of induction.

First, the action of low temperatures was tested on the initial differentiated stromal cells present in suspension. The photocolorimetric analysis of cell populations has shown that, after cooling initial BMSCs at −20°C, the fraction of cells capable of further growth in culture remained about 70%. Under the same conditions of cooling BMSCs differentiated in the adipogenic and osteogenic directions, the fraction of viable cells decreased to 50% of the total number of cells.

Under the action of a lower temperature (−40°C), no significant differences were found in the stability against cold between the differentiated and undifferentiated BMSC. The fraction of cells capable for growth in culture in all variants amounted to 40% of the initial amount.

A further decrease in temperature to −50°C led to cell death. The cells completely lost their ability to spread and proliferate (Fig. 2a). In the same regime of cooling, BMSCs present in sediment retained a higher number of both differentiated and undifferentiated cells able to grow in culture than BMSCs in the state of suspension.

The results of photocolorimetric analysis show that, after cooling initial BMSCs in sediment form at −20°C, the portion of viable cells was about 90%, while at −40°C, this portion was about 70% of the total amount of cells. At a further decrease in temperature to −50°C, a small amount of the cells in sediment

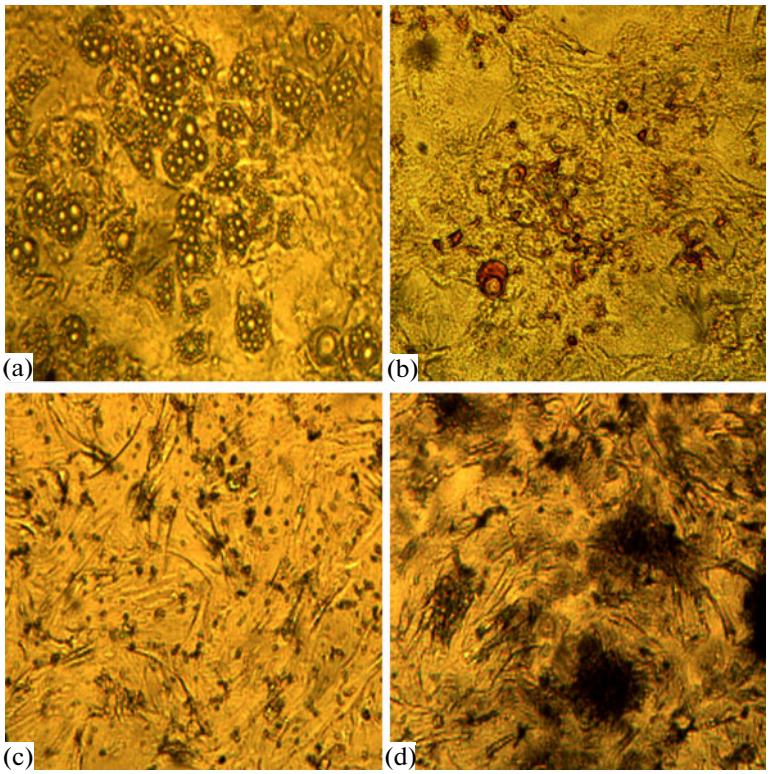

Fig. 1. Bone-marrow stem cells after differentiation in adipogenic and osteogenic directions.
(a, b) phase contrast, (c) histochemical staining of fat droplets with Oil Red, (d) histochemical staining for alkaline phosphatase using the BCIP-NBT mixture. Obj. 10×.

(20%) still survived, whereas all of the cells in suspension died.

Upon cooling BMSCs with adipogenic differentiation, the fraction of viable cells reached 70% after exposure to a temperature of –20°C, whereas at –40°C, 50% of cells remained viable. Upon cooling BMSCs with osteogenic differentiation, cooled in sediment, were less resistant to cold. The fraction of viable cells reached 60% after exposure to a temperature of –20°C, whereas at –40°C 40% of cells remained viable (Fig. 2b). At the same time, in sediment, BMSCs differentiated in both the adipogenic and osteogenic directions, did not survive cooling to –50°C and died.

Thus, the results of the performed studies have shown that stability of BMSCs against the effect of low temperatures decreases after their differentiation in the adipogenic and osteogenic direction.

DISCUSSION

In our previous studies on human epidermis cells, stem and transitory keratinocytes were found to have higher stability against the action of low temperature compared to differentiated cells (Raydan et al., 2011). These differences were preserved in both the culture and skin fragments, which agrees with observations made by cryosurgeons who have treated the skin surface with nitrogen vapor for the purpose of skin rejuvenation. However, the question arises as to whether this stability to cold is a peculiarity of skin cells or if it is also characteristic of other stem and progenitor cells. To answer this question, we studied the degree of stability against the action of low temperatures of BMSCs in their initial state and after directed differentiation in the adipogenic and osteogenic directions.

As shown in the present work, BMSCs differ in their degree of stability against cold depending on their degree of differentiation. After the induction of differentiation in the adipogenic or osteogenic direction, cells in the form of a suspension become less stable against cold. However, at a decrease of temperature to –40°C the portion of remained viable cells, both differentiated and undifferentiated, decreases to 40%. Most likely, this might be because, in both initial and differentiated BMSCs, in the chosen cooling regime, the same protective cell systems preserved in the process of inducing differentiation are impaired. Therefore, the selective effect of cooling at –40°C was not observed.

On the other hand, the cells present in the form of sediment survived lower cooling temperatures than cells cooled in suspension. Furthermore, the higher stability of some undifferentiated BMSCs to cold

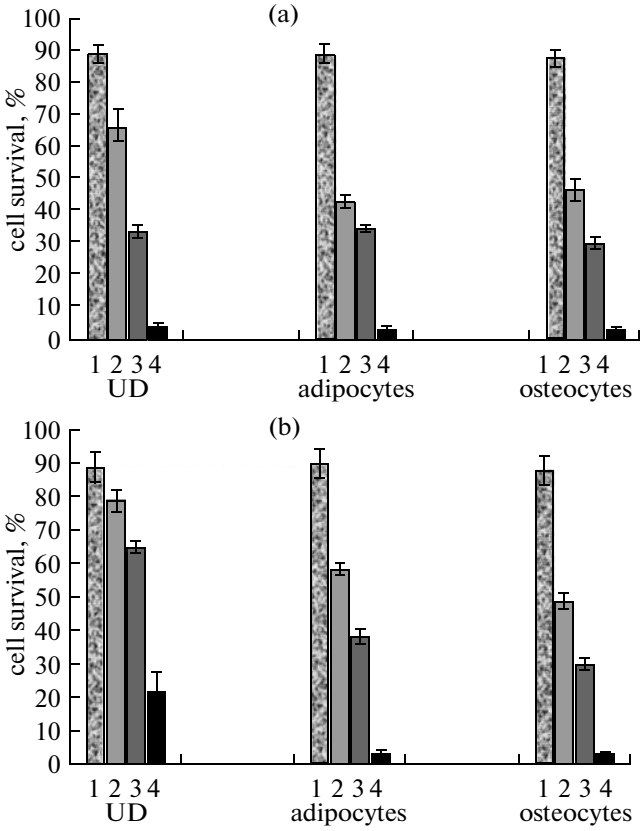

Fig. 2. Survival of undifferentiated (UD) and differentiated BMSC after their cooling to low temperatures. Cells cooled in (a) suspension and (b) sediment. 1. Control temperature 37°C; temperature of cooling: 2. –20, 3. –40, and 4. –50°C.

(–50°C) confirms our suggestion about the change in the stability of BMSCs after their differentiation.

The insignificant difference in stability to cold between the adipogenic and osteogenic cells at their cooling in the form of sediment can be due to different degrees of change in protective mechanisms at the induction of cell differentiation in different directions. A comparative study of the stability of osteocytes and adipocytes requires a more detailed analysis.

Results of the present study agree completely with our earlier data on the increased stability against action of cold of stem and transitory keratinocytes compared to differentiated cells (Raydan et al., 2011). However, the degree of stability to the action of low temperatures in thewe two types of cells is different.

Thus, for instance, the optimal regime of cooling for the keratinocyte suspension amounted to –40°C for 5 min and, for the BMSC suspension, it was –20°C for 5 min, whereas for differentiated BMSC the temperature should be even higher. Even greater differences in stability are observed in these two types of cells during cooling, not in suspension, but in sediment, where they can interact with each other. Thus, keratinocytes in sediment survive a lower temperature regime (–70°C, 5 min) than BMSCs in the same state (–50°C, 5 min). These differences in the degree of stability are quite understandable, since different cells that depend on the type of tissue and their localization in the body are submitted to different effects of changes in the environmental temperature and, therefore, have different degrees of adaptation to low temperatures.

Thus, our obtained results allow us to claim that the stem cells have increased stability to cold compared to differentiated cells. Both the cooling regime and state in which the cells are submitted to cooling are of great significance (Fig. 3). Unlike numerous works dealing with studies of cellular processes in hyperthermia, there are relatively few works on the cell reaction to hypothermia (Holland et al., 1993). It is not easy to study the cell's resistance to cold, since it is not controlled by a certain gene and is associated with complex different factors (Beck et al., 2004), including the degree of the hydrophilic (or hydrophobic) state of the cell membrane (Scott et al., 2005, Byrant et al., 1989; Wolfe, 2002), the change in the cell structure as a result of irregular osmotic stress during cooling (Batysky et al., 1997), the formation of ice crystals inside and outside the cell (Guenther et al., 2006, Mazur et al., 2008), reversible and irreversible protein denaturation

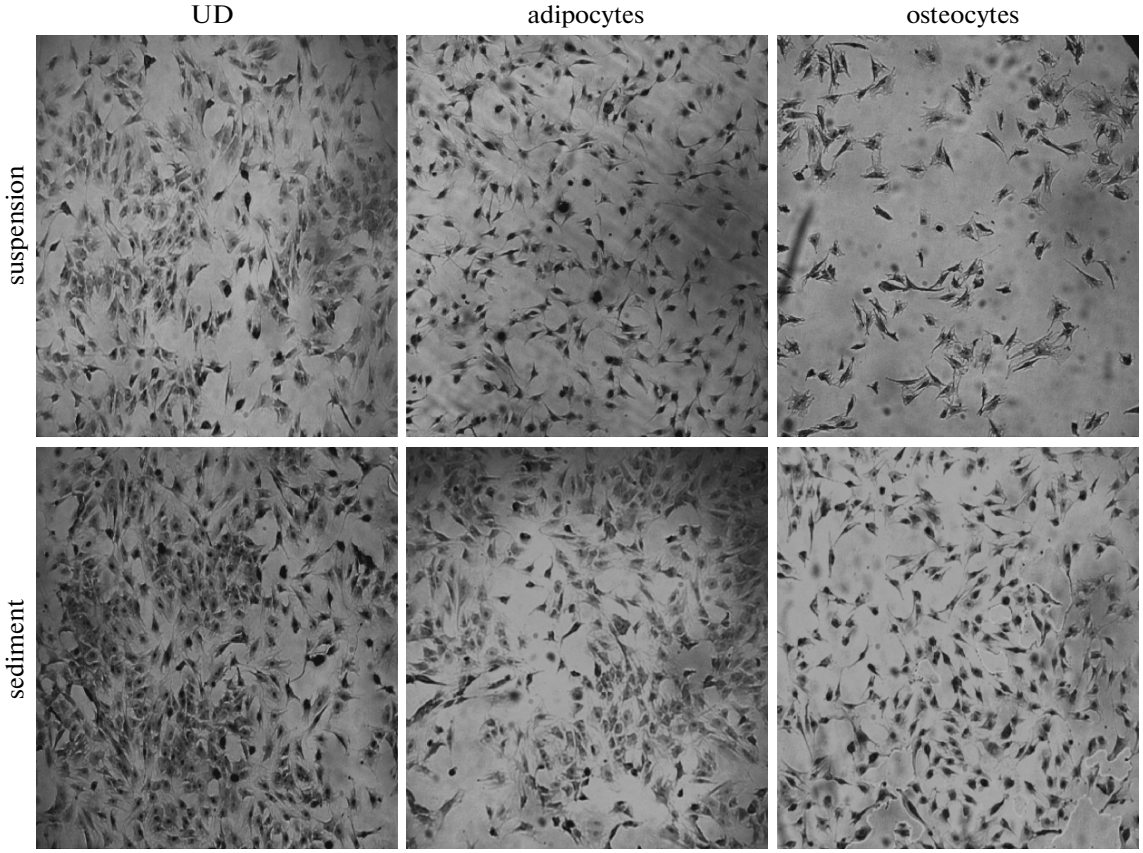

Fig. 3. Microphotograph of differentiated and undifferentiated (UD) BMSC preserved after cooling. It can be seen that density of cells at cooling to –40°C sharply decreases in all variants. Obj. 10×.

(Bischov et al., 2005), changes in the synthesis and localization of intracellular high molecular cryoprotectors (Breton et al., 2000), heat shock proteins (HSP 70) in hypothermia, (Alegna et al., 2005), and cell dehydration (Curry et al., 1994).

In the present work it was determined that the degree of differentiation in BMSC cells also play an important role in their stability to the brief action of low temperatures. To understand the causes in the increased stability of stem cells to cold, it is extremely important to determine which of the above-enumerated factors are important for the change in BMSC stability during differentiation.

REFERENCES

Alegna, R., Paola, T., Guillermo, A., and Strauss, M., Is Hypothermia a Stress Condition in HepG2 Cells? Expression and Localization of Hsp 70 in Human Hepatoma Cell Line, *Tissue Cell*, 2005, vol. 37, pp. 59–65.

Batycky, R.P., Hanmerstedt, R., and Edwards, D.A., Osmotically Driven Intracellular Transport Phenomena, *Phill. Trans. R. Soc. Lond.*, 1997, vol. 355, pp. 2459–2488.

Beck, E.H., Heim, R., and Hansen, J., Plant Resistance to Cold Stress: Mechanisms and Environmental Signals Triggering Frost Hardening and Dehardening, *J. Biosci.*, 2004, vol. 29, pp. 449–459.

Bischof, J., and He, X., Physical Aspects of Cryobiology, *Ann. N.Y. Acad. Sci.*, 2005, vol. 1066, pp. 1–22.

Breton, G., Danyluk, J., Quellet, F., and Sarhan, F., Overexpression of the Acidic Dehydrin WCOR410 Improves Freezing Tolerance in Transgenic Strawberry Leaves, *Annu. Rev.*, 2000, vol. 6, pp. 57–99.

Byrant, G., and Wolfe, J., The Interaction and Fusion of Bilayers Formed from Unsaturated Lipids, *Eur. Biophys.*, 1989, vol. 16, pp. 369–374.

Curry, M.R., Millan, J.D., and Watson, P.F., Cloning and Functional Expression of a New Water Channel Abundantly Expressed in the Testis Permeable to Water, Glycerol, and Urea, *Biol. Reprod.*, 1994, vol. 51, pp. 1014–1021.

Guenther, J.F., Seki, S., Kleinhans, F.W., Edashige, K., Roberts, D.M., and Mazur, P., Extra- and Intra-Cellular Ice Formation in Stage I and II Xenopus Laevis Oocytes, *Cryobiology*, 2006, vol. 52, pp. 401–416.

Holland, D., Roberts, S., Wood, E., and Cunliffe, W., Cold Shock Induces the Synthesis of Stress Proteins in Human Keratinocytes, *J. Invest. Dermatol.*, 1993, vol. 101, pp. 196–199.

Mazur, P., *Physical and Chemical Factors Underlying Cell Injury in Cryosurgical Freezing*, USA. Cryosurgical Treatment: Springfield, 1998.

Mazur, P., and Kleinhans, F.W., Relationship between Intracellular Ice Formation in Oocytes of the Mouse and *Xenopus* and the Physical State of the External Medium—a Revisit, *Cryobiology*, 2008, vol. 56, pp. 22–27.

Mazur, P., The Role of Intracellular Freezing in the Death of Cells Cooled at Supraoptimal Rates, *Cryobiology*, 1977, vol. 14, pp. 251–272.

McGrath, J.J., and Cravalho, E.G., HeLa Cell Freezing Injury: Intracellular iIce Formation Injury and Survival as Function of Coolong Velocity, *Cryobiology*, 1974, vol. 11, pp. 549–556.

Raidan, M., Shubin, N.A., Blinova, M.I., Prokhorov, G.G., and Pinaev, G.P., Effect of Cooling to Low Temperatures on Viability of Human Skin Keratinocytes at Different Stages of Differentiation, *Tsitologiia*, 2011, vol. 53, no. 1, pp. 22–30.

Reyes, M., Lund, T., Lenvik, T., Aguiar, D., Koodie, L., and Verfaillie, C.M., Purification and ex vivo Expansion of Postnatal Human Marrow Mesodermal Progenitor Cells, *Blood*, 2001, vol. 98, pp. 2615–2625.

Scott, K.L., Lecak, J., and Acker, J.P., Transfusion, *Med. Rev.*, 2005, vol. 19, pp. 127–142.

Teryman, H.T., Freezing Injury and Its Prevention in Living Cells, *Annu. Rev. Biophys. Biochem.*, 1974, vol. 3, pp. 341–363.

Wolfe, T., Cellular Thermodynamics, *Encyclopedia of Life Science*, 2002, vol. A1363, pp. 1–17. L