Acta Veterinaria (Beograd), Vol. 60, No. 1, 3-14, 2010.

DOI: 10.2298/AVB1001003K UDK 619:612.419

CULTIVATION OF HAMSTER BONE MARROW HAEMATOPOIETIC STEM AND PROGENITOR CELLS

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(Received 15th September 2009)

Hamster, a hibernating animal, is an important experimental model in research on the influence of hypothermia on different physiological processes. A simple procedure for cultivation and identification of hamster hematopoetic stem cells (HSC) and hematopoetic progenitor cells (HPC) is a premise for a succesfull investigation upon hypothermia effects on hematopoiesis. The aim of this work was to evaluate the utilization of commercially available methylcellulose media (MC) and recombinant mouse and human cytokines for hamster HSC and HPC assays, in order to enable further studies on these cells. Hamster bone marrow mononuclear cells (BMMNC) were plated in MC containing cytokines that support mouse or human HPC growth. Also, BMMNC were resuspended in cytokine supplemented liquid media and incubated for 5 weeks with a four day monitoring of viable cell number. We demonstrated that hamster hematopoietic progenitor cells committed for erythroid lineage and myeloid lineage successfully formed recognizable colonies in both mouse and human MC, while multipotent progenitor cells formed colonies only in mouse MC. We also defined conditions for the evaluation of hamster HSC activity in liquid cultures, based on continuous 5 weeks HSC proliferation. The obtained results verify the utilization of mouse specific MC for further research on hamster HPC biology during hypothermia.

Key words: hamster, hematopoiesis, progenitor cell, stem cell, commercial methylcellulose media

INTRODUCTION

Hamster and other mammalian hibernators offer the unique opportunity for studying what is nature's version of hematopoietic and other tissues preservation during cold exposure inducing state of controlled hypothermia (Carey *et al*., 2003). This "hamster model" is precious for many fields of application, from *in vitro*

cell conservation (Jeanne *et al*., 2009) to research in space flight biology and medicine. With that respect, availability of a reliable *in vitro* system enabling the functional approach to the heterogenous progenitor cells population is essential.

The main *in vitro* approach used in HSCs research, relies on their high proliferative potential (and ability to self renew). These HSCs capacities are expressed in a liquid culture system enabling HSC to proliferate for several weeks or even months (long term culture initiating cells: LTC-IC), producing a large number of HPCs and precursor cells. On the contrary, HPCs have more limited proliferative potential, but are able to form colonies of precursor cells in semisolid media. Previously, growth of hamster CFU-GM, BFU-E and CFU-Mix was demonstrated using condition media (CM) (Eastment *et al*., 1982; Eastment and Ruscetti, 1985) containing undefined activity of species specific mixture of cytokines. These CM were usually prepared using mytogen stimulated spleen cells (Eastment and Ruscetti, 1985) or peripheral blood mononuclear cells (MNC), demanding the continuous testing of CM activity for optimal HPC growth (Kovačević *et al*., 1999). The major advantage of currently existing commercial culture media with recombinant cytokines (as compared to laboratory prepared CM) is the reproducibility of results and time and material sparing. Among commercially available recombinant cytokines recognized to be important in the regulation of hematopoiesis are mainly recombinant human (rh) and mouse (rm) cytokines. Actually, synergistic action of rh or rm interleukin-3 (IL-3), stem cell factor (SCF), interleukin-6 (IL-6), granulocyte-colony stimulating factor (G-CSF) or granulocyte monocyte-colony stimulating factor (GM-CSF) are widely used for HPC and HSC stimulation. However, limiting factor for their exploitation in animal models other than mouse (or human) is the species specificity of cytokines such as IL-3 (Dorssers *et al*., 1987).

We supposed that commercial MC designed to support mouse HPC growth (contain mouse and human recombinant cytokines) will stimulate the growth of hamster HPC. We first tested the growth of hamster progenitor cells derived colonies: CFU-GM, BFU-E and CFU-Mix in mouse MC and afterwards in human MC (contains only human recombinant cytokines). We also compared the growth of mouse and hamster progenitor cells in mouse MC. RhG-CSF was previously successfully used for *in vivo* stimulation of hamster bone marrow cells (Cohen *et al*., 1987) as well as for *in vitro* mouse colony growth (Ivanović *et al*., 1999). Thus, we tested the effect of various doses of rhG-CSF on hamster CFU-GM growth in mouse MC. Finally, we used a combination of mouse and human cytokines to establish hamster BMMNC liquid cultures that could be used for evaluation of an activity reflecting a HSC subpopulation exhibiting a relatively high proliferating potential.

MATERIAL AND METHODS

Animals

The experiments were performed in late spring. Twelve weeks old male and female Syrian golden hamsters (16 animals) and Swiss mice (12 animals) were used in the experiment. Animals were euthanized by cervical dislocation (AVMA

Guidelines on Euthanasia). A pool of BM cells from two animals was always used in the single experiment.

Isolation of mononuclear cells

Femoral and tibial bone marrow (BM) cells were flushed out with 4 mL RPMI 1640 (PAA) containing 20% horse serum (HS) (Sigma) and 20 μL/mL recombinant human deoxyribonuclease (Pulmozyme: Roche Diagnostic) added to prevent cell clumping. Cells were centrifuged on Ficoll density gradient (ratio 3:1) (Biochrom AG) for 25 minutes at 2200 rpm. The hamster or mouse BMMNC were harvested, washed twice and resuspended in RPMI 1640 with 20% HS. The viable cells were counted manually (haemocytometer) using trypan blue dye exclusion test.

Committed progenitor cells assay

BMMNC aliquots (2.5×10^3, 5×10^3 and 10×10^3 cells) were seeded in 1 mL (in duplicate) of mouse or human specific, standardized mouse or human specific semi solid MC media, in 35 mm Petri dishes:

Mouse specific medium: Methocult GF M3434 with 50 ng/mL rm SCF, 10 ng/mL rmIL-3, 10 ng/mL rhIL-6, 3 units/mL rhEpo; or *Methocult GF M3534* (as *Methocult GF M3434* but without rhEpo) (Stem Cell Technologies).

Human specific medium: Methocult GF H4434 with 50 ng/mL rhSCF, 10 ng/mL rhGM-CSF, 10 ng/mL rhIL-3 and 3 units/mL rhEpo (Stem Cell Technologies).

Some assays were performed with addition of 10, 100 or 1000 ng/mL rhG-CSF (Neupogen, Amgen-Roche) to *Methocult GF M3534*.

After 7 days of culture [according to Eastment and coworkers (1982)] at 37°C in a humidified atmosphere with 20% O_2, and 5% CO_2 colony number was enumerated under an inverted microscope. CFU-GM colonies were detected as groups with more than 50 cells radially dispersed around the central colony area. BFU-E colonies were recognized as large bursts of fully hemoglobinised (and thus red) multiple cell clusters. Large colonies containing visually recognizable two types of cells: white cells and multiple red cell clusters were designated as CFU-Mix colonies. Cells from colonies were transferred on glass slides and cytology smears were stained with Hemacolor (Merck).

Liquid culture assay

HSC are primitive pluripotent cells. When appropriately stimulated, the HSC proliferate and differentiate, forming committed progenitors and further on, a large number of precursor cells thus enabling long last expansion of the total cell number in liquid culture. Herein, we wanted to establish a liquid culture that would enable the proliferation of HSC, reflecting their activity among tested cells (Ivanović *et al*., 2000). Hamster BMMNC (80×10^3 cells per mL) were resuspended in RPMI 1640 (PAA) with 20% horse serum (HS, Sigma) and different cytokine combinations: 1. rmSCF, rmIL-3, rhIL-6; 2. rmSCF, rmIL-3, rhIL-6, rhG-CSF; 3. rmSCF, rmIL-3, rhIL-6, rhG-CSF and rh megakaryocyte growth and development factor (MGDF). Cytokines were used in following doses: 50 ng/mL rmSCF, 10 ng/mL rmIL-3, 10 ng/mL rhIL-6 (all from Stem Cell Technologies), 20 ng/mL rhG-

CSF (Neupogen, Amgen-Roche), 50 ng/mL rh MGDF (Amgen). Some liquid culture assays were performed without cytokine supplementation. Duplicate of 1.5 mL liquid cultures were established in 24-well plates and every 4 days viable cell were counted with trypan blue. At the same time, demi-depopulation and addition of fresh cytokine-supplemented medium was performed, that was taken into account for normalization of the total cell number.

Data analysis

Mean values (±SE) were calculated from data of at least 3 independent experiments. Differences between the variables were assessed using Student's *t* test.

RESULTS

Commercial media optimized for murine haematopoietic cells supports hamster CFU-GM, BFU-E and CFU-Mix growth

Plating efficiency for hamster CFU-GM and BFU-E in mouse and human MC, respectively (Figure 1: A and B) was the same and followed the linearly increasing number of BMMNC. However, only mouse specific Methocult GF M3434 supported the growth of hamster CFU-Mix (Figure 1: C). Also, BFU-E colonies formed in Methocult GF M3434 were intensively red colored, large and multicentric (Figure 2: A). Smears made from these colonies contained erythroblasts at different maturation stages (Figure 2: C and D). On the contrary, BFU-E colonies formed in human specific Methocult GF H4434 were small and unremarkable (not shown).

The majority of CFU-GM formed in both types of MC had between few hundred and few thousand cells (Figure 2: B) containing myelocytes and even band granulocytes (Figure 2: E and F). About 20% of CFU-GM colonies (*Methocult GF M3434*) had the appearance of very dense colonies (containing millions of cells). CFU-Mix colonies were very large (diameter more than 2 mm) and contained cells of erythroid and myeloid lineage. The distribution of colonies that enabled the best and easiest colony scoring in both types of MC was found when 5×10^3 MNC were plated.

As hamster and mouse are filogenetically related species we considered interesting to observe similarities and differences on their colony growth in mouse specific *Methocult GF M3534*. The number of mouse CFU-GM was significantly higher at all three BMMNC concentrations plated (Figure 1: D). Also, mouse CFU-GM had a higher proliferative potential, majority of them having millions of cells and forming large uniformly sized and shaped colonies. This important difference in number and size of hamster and mouse colonies in mouse specific *Methocult GF M3534* directed us to test the effect of rhG-CSF on plating efficiency and size of hamster CFU-GM. Our results demonstrated that addition of 10 ng/mL, 100 ng/mL and 1000 ng/mL of rhG-CSF in *Methocult GF M3534* only slightly increased the plating efficiency of hamster CFU-GM (Figure 1: E) and had only a moderate effect on colony size.

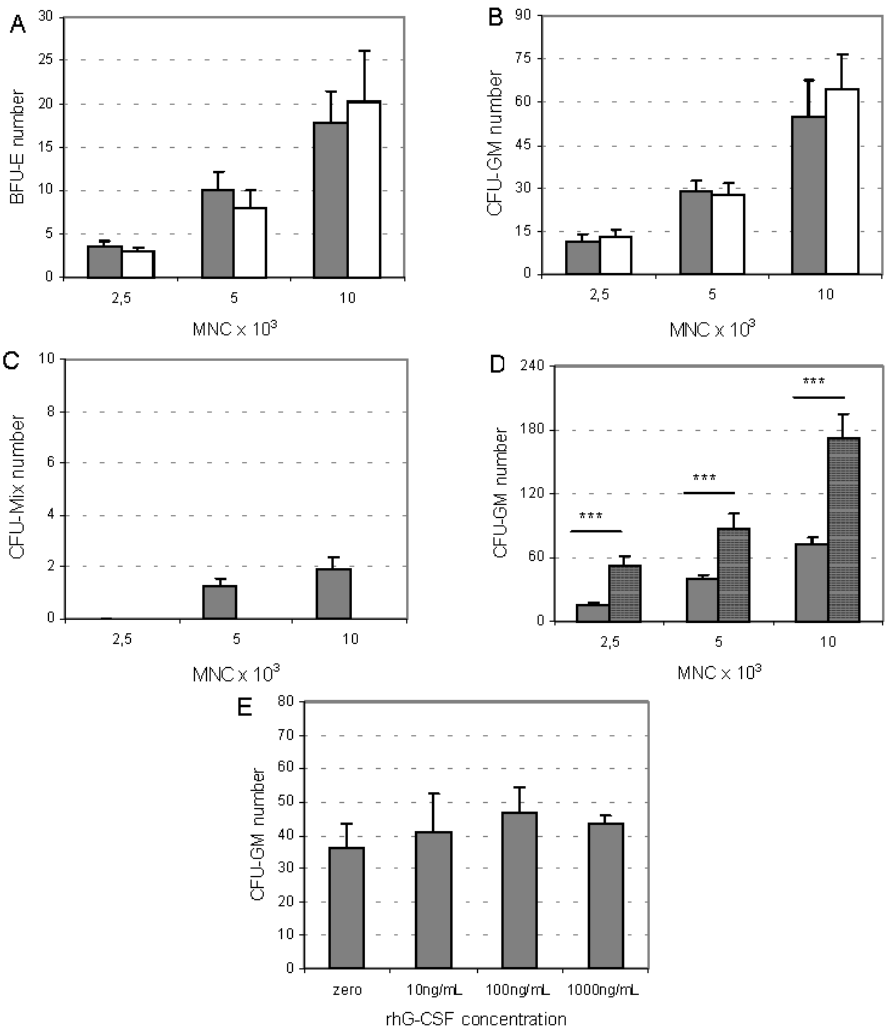

Figure 1. Plating efficiency of hamster bone marrow committed progenitor cells in different methylcellulose (MC) media. Data are presented as mean number (±standard error) of 6 experiments.
Number of hamster burst forming unit-erythrocyte (BFU-E) colonies (A), colony forming unit granulocyte-monocyte (CFU-GM) colonies (B) and CFU-Mix colonies (C) plated in human specific *Methocult GF M4434* (white bars) and in mouse specific *Methocult GF M3434* (gray bars). Number of mouse CFU-GM colonies (hatched bars) and hamster CFU-GM colonies (gray bars) plated in mouse specific *Methocult GF M3534* (D). Effect of different rhG-CSF concentrations on number of CFU-GM colonies plated in mouse specific *Methocult GF M3534*, at concentration of 5 x 10^3 cells/mL (E).
Abbreviation: MNC – mononuclear cells

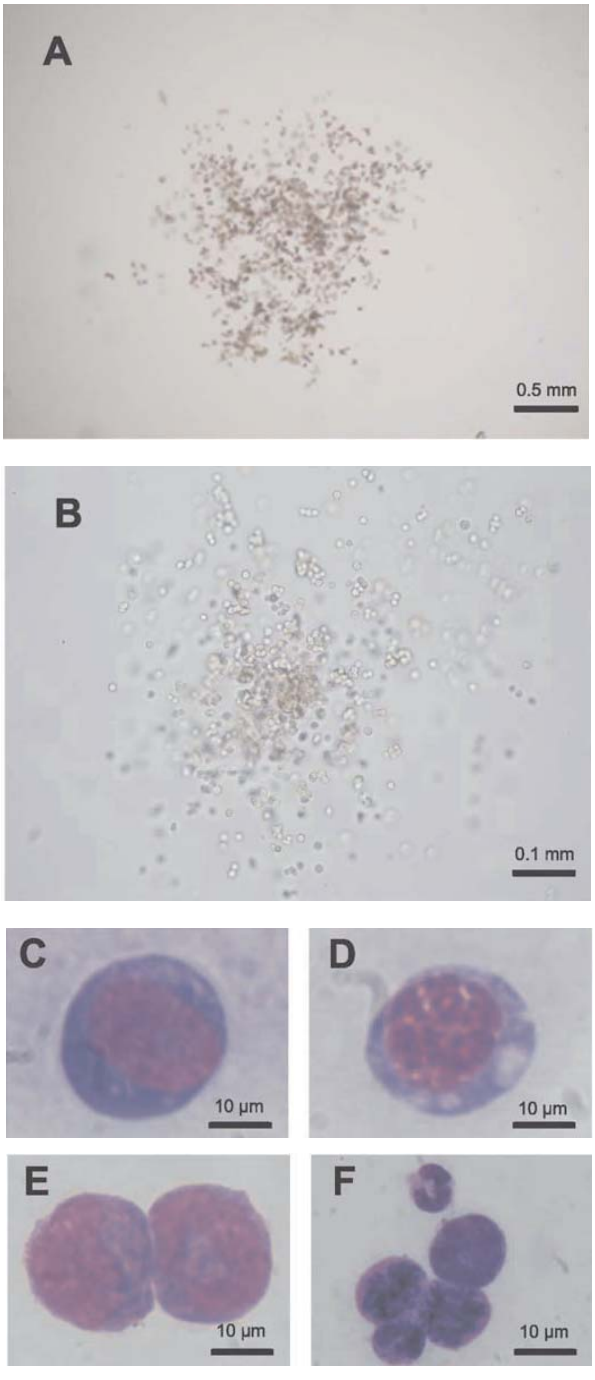
A
0.5 mm
B
0.1 mm
C
10 µm
D
10 µm
E
10 µm
F
10 µm

Figure 2. Hamster burst forming unit-erythrocyte (BFU-E) and colony forming unit granulocyte-monocyte (CFU-GM) colonies by day 7 of cultivation, plated in *Methocult GF M3434* (A and B respectively, magnification 100 x and 400 x). Cytology smears of BFU-E and CFU-GM colonies. Erythroblasts at different maturation stages from BFU-E colony by day 7 of cultivation (C and D magnification 1000 x). Myeloblasts at different maturation stages from CFU-GM colony by day 7 of cultivation (E and F, magnification 1000 x). Hemacolor staining.

Liquid culture with a combination of mouse and human recombinant cytokines supports proliferation of hamster HSC

Combination of cytokines that stimulated hamster bone marrow HSCs to proliferate for at least 5 weeks contained rmSCF, rmIL-3, rhIL-6, rhG-CSF and rhMGDF (Figure 3). With this combination of cytokines, after 20 days of linear increase, the total cell number was multiplied approximately 40 times and the reached level was sustained for another 16 days (Figure 3). Thereafter the cultures were stopped. Two restrictive cytokine combinations (SCF, IL-3, IL-6 or SCF, IL-3, IL-6 + G-CSF) stimulated only short term HSC proliferation (8 days and 16 days duration, respectively), without a significant expansion of the total cell

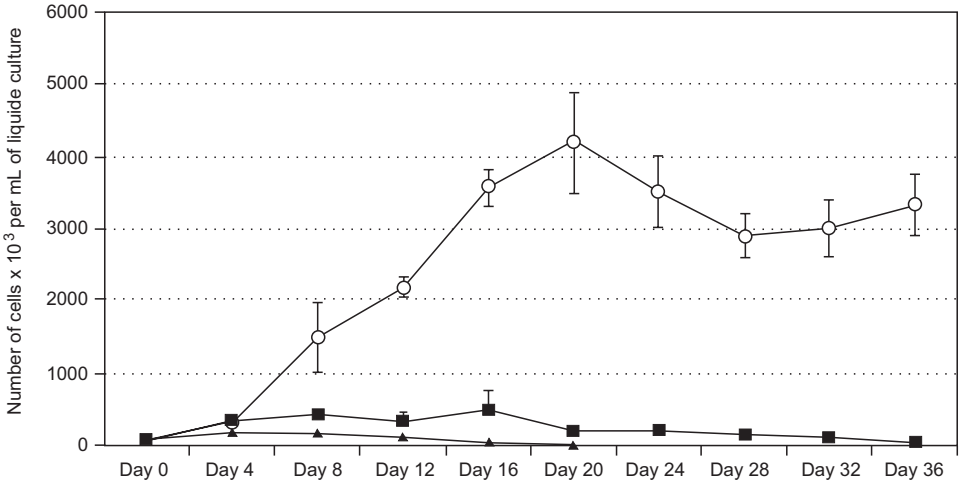

Figure 3. Mean number (±standard error) of nucleated cells during 36 days of bone marrow mnononuclear cell (BMMNC) liquid culture with 20% horse serum and combination of three cytokines: rmSCF + rmIL-3 + rhIL-6 (-▲ -); four cytokines: rmSCF + rmIL-3 + rhIL-6 + rhG-CSF (-■ -); five cytokines: rmSCF + rmIL-3 + rhIL-6 + rhG-CSF + rhMGDF (-O -) according to the concentration described in material and method section. Every 4 days viable cells were counted and half of medium was replaced with fresh cytokine supplemented medium.
Abbreviations: rmSCF – recombinant mouse stem cell factor; rmIL-3 – recombinant mouse interleukin-3; rhIL-6 – recombinant human interleukin-6; rhG–CSF – recombinant human granulocyte-colony stimulating factor: rh MGDF – recombinant human megakaryocyte growth and development factor

number (Figure 3). In addition, in either MGDF free cytokine combination, after reaching the plateau, the total cell number declined progressively (Figure 3). In the absence of cytokines, cell survival longer than 4 days was not detected (data not shown).

DISCUSSION

This work, for the first time, describes hamster bone marrow HPC growth in mouse specific MC and defines the cytokine combination for successful HSC proliferation in a BMMNC liquid culture.

We demonstrated that plating of hamster BMMNC in mouse specific MC3434 resulted in reproducible and reliable growth of committed progenitor cells derived colonies: BFU-E, CFU-GM and CFU-Mix. We also demonstrated that plating efficiency of hamster BFU-E and CFU-GM was the same, but the BFU-E colony size was reduced in human specific Methocult GF H4434 comparing to mouse specific Methocult GF M3434. Recently, it was also reported that bovine and swine bone marrow CFU-GM were successfully cultured in complete human specific MC (Keller *et al*., 2004; Gomez-Ochoa *et al*., 2007) and that horse BFU-E were stimulated with feline cytokines (Swardson and Kociba, 1996). This phenomenon is curious knowing that IL-3, previously called multi colony stimulating factor, indispensable for the development of different hematopoietic cell populations, is species specific (Dorssers *et al*., 1987; Burger *et al*., 1990; Stevenson and Jones, 1994). However, our results also demonstrated that both combinations of cytokines (present in mouse and human specific MC) supported the growth of hamster bone marrow CFU-GM and BFU-E colonies. However, combination of rmIL-3, rmSCF, rhIL-6 and rhEpo (*Methocult GF M3434*) was able to stimulate proliferation and maturation of BFU-E giving birth to large multicentric colonies, while the combination of rhIL-3, rhSCF, rhGM-CSF and rhEpo (*Methocult GF H443*) primarily had the effect on survival of these cells resulting in growth of small unremarkable colonies. Gómez-Ochoa *et al*. (2006) demonstrated that *Methocult GF H4435* containing rhIL-3, rhSCF, rhGM-CSF, rhIL-6, rhG-CSF and rhEpo enabled growth of high number of CFU-Mix from hamster blood MNC. These findings emphasized a very important role of IL-6 and/or G-CSF for proliferation of primitive erythroid progenitors in hamsters. We supposed that human specific *Methocult GF H4434* (used in our experiment: rhSCF, rhGM-CSF, rhIL-3 and rhEpo) did not support the CFU-Mix formation, probably because their high proliferative potential and potential for differentiation could not have been expressed without IL-6 and/or G-CSF. It is interesting to note that while supporting hamster and rat progenitor colony growth, human specific *Methocult GF H4435*, failed to support mouse progenitor colony growth (Gómez-Ochoa *et al*., 2006), pointing to higher evolutionary divergence of certain mouse hematopoietic growth factors comparing to hamster, rat and human. Also, circumvention of IL-3 species specificity and successful colony growth in all examples noted, could be a consequence of synergistic and redundant action of other cytokines known to be cross-reactive.

Comparison of mouse and hamster CFU-GM growth revealed that a mean number of hamster colonies was significantly lower, and their colony size was smaller in mouse specific MC than the number and size of mouse colonies. We assumed that the observed difference issued from lower plating efficiency of hamster CFU-GM comparing to mouse CFU-GM and not from their absolute lower number. It was previously reported that hamsters receiving s.c. injection of rhG-CSF had an increase in the number and cycling activity of BM CFU-GM and an increase in the absolute number of granulocytes (Cohen *et al.*, 1987). To test the hypothesis that plating efficiency of hamster CFU-GM in mouse MC could be improved by addition of rhG-CSF we used different rhG-CSF concentrations. Unexpectedly, rhG-CSF did not alter the plating efficiency of hamster progenitor cells, meaning that its target cells are either more primitive or more mature hematopoietic cells that are not characterized by colony forming ability. The other explanation could be that *in vitro* test system was missing some synergistic molecules important for G-CSF activity. It is interesting that addition of 100 ng/mL of rbG-CSF in MC4534 (MC containing human cytokines) with bovine bone marrow cells, increased the number of mature day 3 CFU-GM, but did not affect the number of day 6, day 7 and day 8 CFU-GM (Keller *et al*., 2004), giving evidence that relatively mature progenitor cells are targets for G-CSF.

First *in vitro* investigations on hamster HSC have been done by Eastman *et al.* (1982). They demonstrated that, opposite to all other mammalian species investigated, hamster HSC do not need a feeder layer to survive, proliferate and differentiate for over 6 months in long term suspension (LTS) cultures. These LTS cultures were maintained without cytokine supplementation, but were serum dependent (Eastman *et al*., 1982). Our liquid cultures, despite being serum supplemented, were dependent on the presence of cytokine combinations (rmIL-3, rmSCF, rmIL-6, rhG-CSF, rhMGDF). They were successfully maintained for 5 weeks without signs of cell number decline giving evidence that high proliferative potential of HSC was expressed. Cytokine dependence of our liquid cultures could be related to MNC isolation step responsible for a significant concentration of HPC, but loss of different cell populations possibly involved in maintenance of long term suspension cultures without cytokine supplementation. The accessory cells effects on the expansion of HSC in liquid cultures was previously described to be dependent on the cytokine combination used (Xu *et al*., 2000). It is interesting to note that in our liquid cultures, MGDF had an important impact on expression of HSC proliferative potential enabling a significant expansion of total cell number (40 times). This finding could be explained with a fact that MGDF, aside of being a potent stimulator of megakaryocyte lineage, is among the most potent stimulators of primitive HSC, used in recently reported *ex vivo* human HSC expansion protocols (Ivanović *et al*., 2006; McNiece *et al*., 2000). Moreover, effectiveness of rhMGDF exerted on hamster HSC proliferation is in line with finding that MGDF cDNAs were highly conserved among human, canine, and murine species (Bartley *et al*., 1994). Nonetheless, we consider that sustainable proliferation of cells obtained in our liquid cultures offers a simple test for HSC activity. This test is of interest when the activity of HSC exposed to different

conditions has to be compared (Ivanović *et al*., 2000; Kovačević Filipović *et al*., 2007).

In conclusion, comparative approach in studying physiology of hematopoietic cells enables the distinction of conserved and adaptive body responses (Powell, 2003). Moreover, the possibility to *in vitro* evaluate heterogeneous hematopoietic cells gives us a good opportunity to use the hamster (and hamster bone marrow cells) as an excellent model for the investigation of mechanisms enabling hematopietic cell survival during hypothermia.

ACKNOWLEDGMENT:
This study was funded by a grant number 145048 from the Ministry of Science and Environmental Protection, Republic of Serbia.

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KULTIVACIJA MATIČNIH I PROGENITORSKIH ĆELIJA HEMATOPOEZE IZ KOSTNE SRŽI HRČKA

KOVAČEVIĆ FILIPOVIĆ MILICA, OKIĆ IVANA, PETRIĆEVIĆ TANJA, MOJSILOVIĆ S, KRSTIĆ ALEKSANDRA, JOVČIĆ GORDANA, BUGARSKI DIANA, MILENKOVIĆ P, PETAKOV MARIJANA, RADOVANOVIĆ ANITA, BOŽIĆ TATJANA i IVANOVIĆ Z

SADRŽAJ

Fiziološka hibernacija u koju hrčci ulaze prilikom izlaganja niskim temperaturama, čini ove životinje zanimljivim eksperimentalnim modelom za ispitivanje hematopoeze u uslovima hipotermije. Preduslov za ovo ispitivanje je postojanje jednostavne metode za kultivaciju i identifikaciju hematopoetskih ćelija hrčka. Cilj ovog rada je bio da se ispita mogućnost kultivacije progenitorskih ćelija hematopoeze hrčka u kompletnoj metil celulozi dizajniranoj za kultivaciju mišijih i humanih hematopoetskih ćelija, kao i da se odrede optimalni uslovi za kultivaciju matičnih ćelija hematopoeze hrčka u tečnoj kulturi. Mononuklearne ćelije kostne

srži hrčka su posađene u metil celulozu i u tečnu kulturu. Oba medijuma su sadržala kombinacije rekombinantnih mišijih i/ili humanih citokina. Kolonije progenitorskih ćelija opredeljenih za mijelopoezu i opredeljenih za eritropoezu su se formirale u metil celulozi dizajniranoj za kultivaciju mišijih i humanih hematopoetskih ćelija, dok su se primitivnije kolonije sastavljene od oba tipa ćelija (mijeloidna i eritrocitna loza) formirale samo u metil celulozi dizajniranoj za kultivaciju mišijih hematopoetskih ćelija. Osim toga, populacija matičnih ćelija hematopoeze hrčka je proliferisala u tečnim kulturama tokom 5 nedelja bez znakova opadanja proliferativnog potencijala. Ova istraživanja pokazuju da se primenjene metode mogu uspešno koristiti za ispitivanje hematopoeze kod hrčka.