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PIG BONE MARROW AND PERIPHERAL BLOOD ERYTHROID PROGENITOR CELLS IN S PHASE OF THE CELL CYCLE

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The proliferative rate of hemopoietic progenitor cells, i.e. the percentage of cells in the synthetic (S) phase of the cell cycle is a very sensitive parameter for detecting the steady-state misbalance arising in response to stimulatory or inhibitory molecules, or in postmyeloablative repopulation of hemopoiesis. This parameter is also predictive for the sensitivity of these cells to irradiation injury and specific cytotoxic drugs. In spite of the fact that miniature inbred and domestic pig breeds have been used as large animal models in hemopoietic research and in experimental bone marrow transplantation, data concerning the proliferative rate of porcine progenitor cells are still lacking. The aim of this study was to examine the steady-state proliferative rate of bone marrow and peripheral blood erythroid progenitor cells of adult pigs. The percentage of cells in S phase of the cell cycle of both immature, BFU-E (Burst Forming Unit-Erythroid) and mature, CFU-E (Colony Forming Unit-Erythroid) erythroid progenitor cells was determined by the suicide technique based on the proportion of these cells killed after in vitro treatment of the cells with cytosine arabinoside (Ara-C). The results revealed different relative numbers of immature and mature erythroid progenitor cells in S phase of the cell cycle in the bone marrow, namely, 33.3% for BFU-E and 55.1% for CFU-E. In peripheral blood the proliferative rate of BFU-E progenitors was 26.3%. The data obtained show that the proliferative rate of porcine erythroid progenitor cells is similar to the values determined for other mammalian species.

Key words: proliferative rate, CFU-E, BFU-E, pig, in vitro

INTRODUCTION

The proliferative rate of a cell population may be represented as the number of cells in the DNA-synthetic phase of the cell cycle in a defined interval of time (Oehlert, 1973). According to modern concepts, maturing of stem and progenitor cells is associated with the gradual elevation of the percentage of the population

in the active phase of the cell cycle. While primitive categories of stem cells are quiescent, up to 80% of cells of the maturest category of the erythroid lineage, CFU-E, are in an active cell cycle. Most recent investigations indicate that primitive stem cells are in a very slow cell cycle (Bradford et al., 1997) rather than not cycling at all. During the fetal and neonatal period the proliferative rate of hemopoetic progenitor cells is high, but it declines during aging (Peschle et al., 1981; Tejero et al., 1989). However, even in adults, stem and progenitor cells keep a high reserve proliferative potential. For example, CFU-S (colony forming unit-spleen - pluripotent progenitor cells) in steady-state have a proliferative rate of 10%, but with increasing body demands for mature blood cells, it could reach 50% (Lajtha et al., 1969; Milenković et al., 1993).

Cell cycle kinetics is regulated through a network of stimulatory and inhibitory molecules, compensating increased or decreased body demands. Moreover, various pathological processes, like neoplastic transformation of stem or progenitor cells, can directly alter their proliferative activity. Numerous studies have been related to murine and human stem and progenitor cells in S phase in different conditions (Iscove, 1977; Hara & Ogawa, 1977; Gregory & Eaves, 1978; Milenković & Pavlović-Kentera, 1979; Monette et al., 1980; Kubota et al., 1983; Jovčić et al., 1996, Ivanović, 1997). Reports on ovine (Barker, 1980), rat (Kimura et al., 1986; Basara et al., 1988; Ivanović et al., 1995; Ivanović et al., 1995a), canine (Abkowitz et al., 1988) and feline (Linenberger et al., 1991) progenitor cells in the active phase of the cell cycle have been published, too.

In order to estimate the steady-state proliferative rate of bone marrow BFU-E and CFU-E, and peripheral blood BFU-E progenitor cells of adult pigs, a colony forming assay on methyl-cellulose, and the Ara-C suicide technique *in vitro* have been used. Ara-C belongs to the group of S phase specific drugs and its primary cytotoxic action results from incorporation into nucleic material and inadequate ligation of fragments of newly synthesized DNA.

MATERIALS AND METHODS

Animals and cell suspensions

Blood and bone marrow samples were taken from 10 clinically normal industrial breed pigs of both sexes, 4-6 months old. Blood samples (20 ml) were collected from the jugular vein in sterile tubes with preservative-free sterile heparin (40U/ml). The animals were stunned and slaughtered. Bone marrow was harvested by direct surgical curettage from the *ossis ishii (symphysis pelvis)* (the bodies passed through a warm water pool at 62^0C), and the cells were suspended in Iscovežs Modification of Dulbeccožs Medium (IMDM, GibcoBRL, Life Technologies, Paisley, Scotland) and constantly mixed for about one hour.

For all samples standard peripheral blood analysis was done. Mononuclear cells (MNC) were obtained on a Ficoll-Hipac 1.077g/ml density gradient (Lymphoprep, Nycomed, Oslo, Norway). Cell viability was determined before plating using the trypan-blue exclusion test.

Determination of erythroid progenitor cells in S phase of the cell cycle

The Ara-C suicide assay was performed to determine the proportion of progenitor cells in the S phase of the cell cycle, (Shulman & Robinson, 1986). Briefly, $5x10^6$ cells were incubated for one hour with 40(μg of Ara-C (Upjohn Company, Kalamazoo, Michigan, USA) in IMDM, supplemented with 15% fetal

calf serum (FCS, Serva Feinbiochemica, New York, NY, USA) in 2 ml at 37°C in a humidified atmosphere with 5% CO2 in air (+ Ara-C sample). Cells incubated under the same conditions without Ara-C were used as a control (- Ara-C sample). At completion of the incubation period, the cells were washed in IMDM and appropriately diluted suspensions were made to assay BFU-E and CFU-E progenitors. The reduction in colony formation by the cells from samples treated with Ara-C was proportional to the number of progenitors in DNA synthesis (suicide), i.e. represented an indirect measure of the proportion of proliferating progenitor cells.

$$\text{Progenitor cells in S phase (\%)} = \left(1 - \frac{\text{No of colonies in the + Ara-C sample}}{\text{No of colonies in the - Ara-C sample}}\right) \times 100$$

Erythroid colony assays

The colony forming assay for BFU-E and CFU-E was performed according to the original method of Iscove et al., (1974). Briefly, optimal growth conditions for bone marrow BFU-E and CFU-E were $1x10^5$ MNC plated in 1 ml of final mixture containing 0.8% methylcellulose (ICN, Costa Mesa, USA), 30% FCS, 2% bovine serum albumin (BSA, Sigma, St Louis, MO, USA), $2x10^{-5}$ M (mercaptoethanol (ME, Sigma, St Louis, MO, USA) and 6 IU of recombinant human erythropoietin (rhEpo, Elanex Pharmaceuticals, Inc., Bothell, WA, USA) in IMDM. Cultivation of peripheral blood BFU-E required $1x10^6$ MNC and the addition of 5% leukocyte conditioned medium (LCM) in the final culture mixture. The culture mixtures were plated in duplicate and incubated at 37°C in a humidified atmosphere with 5% CO_2 in air: 10 days for BFU-E and 4 days for CFU-E.

Statistical analysis

Data were subjected to descriptive statistical methods including the arithmetic mean and standard error.

RESULTS

All determined peripheral blood parameters were in the physiological range for pigs (data not shown). The viability of separated mononuclear cells before plating was 90-95 % indicating that the procedure used was optimal for the recovery of bone marrow and peripheral blood cells.

The proliferative rates of different categories of erythroid progenitor cells in bone marrow and peripheral blood, determined on the basis of Ara-C suicide in vitro, are presented in Table 1. The assessment of the proportion of progenitor cells in S phase of the cell cycle revealed that in bone marrow the percentage of BFU-E in active cell cycle was lower than the percentage of CFU-E. The cycling status of peripheral blood BFU-E was slightly lower than the values found in bone marrow, while CFU-E progenitors were not detected in pig blood.

The range of actual numbers of erythroid progenitor cells derived colonies in bone marrow and peripheral blood and the range of their percentage in S phase of the cell cycle are presented in Table 2 In bone marrow the number of CFU-E derived colonies had a broader range than BFU-E, but higher variations in the percentage of progenitor cells in S phase among the pigs were observed in BFU-E progenitors. In peripheral blood, a broad range of both the number of BFU-E derived colonies and their percentage in S phase was found.

Table 1. The percentage of pig bone marrow and peripheral blood erythroid progenitor cells in S phase of the cell cycle

PROGENITOR CELLS	% in S phase
Bone marrow	
BFU-E	33.3 ± 5.8
CFU-E	55.1 ± 1.7
Peripheral blood	
BFU-E	26.3 ± 3.8

After incubation of the cells with or without Ara-C, bone marrow BFU-E and CFU-E were cultured in the presence of 6 IU rhEpo and peripheral blood BFU-E in the presence of 6IU rhEpo and 5% LCM. The percentage of progenitor cells in S phase was determined on the basis of Ara-C suicide. The data are shown as mean ± SEM

Table 2. The range of the actual number of erythroid progenitor cell derived colonies and range of their percentage in S phase of the cell cycle

PROGENITOR CELLS	N° of colonies	% in S phase
Bone marrow		
BFU-E	41 - 86	19.8 - 53.6
CFU-E	336 - 1016	50.0 - 60.5
Peripheral blood		
BFU-E	4 - 17	18.2 - 35.7

For comparison of our results obtained in pigs with data published for other mammalian species, values for the proliferative rate of the erythroid progenitor cells are presented in Table 3.

Table 3. The percentage of bone marrow erythroid progenitor cells in S phase of the cell cycle in man and some animal species.

Species	% in S phase BFU-E	% in S phase CFU-E	cytotoxic agent	References
Human	10 ±0.6 pBFU-E; 35 ±1.5 iBFU-E	56 ±4.9	^{3}H-TdR*	Peschle et al., 1981
Dog	27 ±6	64 ±7	^{3}H-TdR	Abkowitz et al., 1988
Cat	25 ±4	44 ±7	^{3}H-TdR	Linenberger et al., 1991
	28 ±2	62 ±4		Abkowitz et al., 1988a
Rat	22		Ara-C	Basara et al., 1988
	19.4	68.2	^{3}H-TdR	Kimura et al., 1986
Mouse	30 ±8.1	76 ±2.7	^{3}H-TdR	Iscove, 1977
	36	74		Hara & Ogawa, 1977
	21.7 ±2.6 pBFU-E; 51.5 ±3 iBFU-E	70.1 ±0,6		Gregory & Eaves, 1978
		76 ±8	HU$^{\sim}_{(in\ vivo)}$	Monette et al., 1980
	22.8 ±4.9			
			Ara-C	Jovčić et al., 1996

*Tritiated thymidine ~Hydroxyurea

The data are shown as mean ± SEM

DISCUSSION

The purpose of this study was to assess the percentage of pig bone marrow and peripheral blood erythroid progenitor cells in S phase of the cell cycle in steady-state, since data concerning the proliferative activity of porcine progenitor cells are still lacking. The results obtained demonstrated different proportions of immature and mature erythroid progenitor cells in S phase of the cell cycle in the

bone marrow and peripheral blood and are in accordance with the majority of numerous reports concerning the proliferative rate of erythroid progenitor cells in other mammalian species, regardless of the different techniques used.

In mice and humans, primitive (pBFU-E) and intermediate BFU-E (iBFU-E) could be distinguished, using the proportion of BFU-E progenitor cells in the active cell cycle as one of the criteria. Murine primitive BFU-E have 20% of the cells in S phase and intermediate 50% (Gregory & Eaves, 1978), while the corresponding values for humans are 10% and 35%, respectively (Peschle et al., 1981). In our experimental conditions 33% of the porcine bone marrow BFU-E population was in S phase of the cell cycle. These progenitors were previously shown not to respond to LCM as an exogenous source of growth factors other than Epo. On the other hand their growth was absolutely Epo dependent as the number drastically declined after postponing Epo addition for only 24 h (Kovačević et al, 1999). Taken together these data imply that bone marrow BFU-E in our experimental conditions belong to the intermediate category of this progenitor cell population.

The percentage of progenitor cells in active phase of the cell cycle has a circadian rhythm, with up to 50% difference throughout each day as determined by incorporation of BrdU (5-bromo-2-deoxyuridine) (Wood et al, 1998). These daily variations are most likely related to the circadian rhythm of the concentrations of hematopoietic stimulatory and inhibitory cytokines and hormones and could be one of the explanations for individual differences in BFU-E proliferation rate in our experiment.

One of the current questions raised on the basis of clinical trials of mobilized peripheral blood stem cells is whether circulating progenitor cells may differ from their bone marrow counterparts. It seems that peripheral blood BFU-E are quiescent with respect to DNA synthesis (Ogawa et al., 1977; Peschle et al., 1981), even in cytokine treated mice or humans. Since this quiescence was not due to inhibitory substances in the blood (Roberts and Metcalf, 1995), it possibly reflects a more primitive state. It is interesting to note that according to some investigators even BFU-E from umbilical cord blood are noncycling cells (Schekhter-Levin et al., 1984), while others report the presence of cycling BFU-E in fetal and neonatal blood (Peschle et al., 1981). In contrast to these data, we have obtained a relatively high proliferative rate for porcine peripheral blood BFU-E. This could be the result of the inaccuracy of the test used, in respect to the small colony number obtained in the colony forming assay and consequently the high percentage of reduction in the colony number after the Ara-C treatment. On the other hand the high proliferative rate could be a result of their different sensitivity to the cytotoxic agent used. Kubota et al., (1983) reported that human BFU-E progenitors from peripheral blood were insensitive to ^{3}H thymidine, moderately sensitive to hydroxyurea, and very sensitive to Ara-C. Our findings are similar to the proliferative rate (36,6%) of a subpopulation of human peripheral blood BFU-E separated on a discontinuous density gradient (Schekhter-Levin et al.,1985).

The observed high proliferative rate of porcine bone marrow CFU-E is consistent with the values obtained in different species. Data showing that CFU-E proliferative rate does not change, or changes only slightly in response to bleeding (stimulation of erythropoiesis) or hypertransfusion (suppression of erythropoiesis) (Iscove, 1977) point to a high proliferative rate as their intrinsic property.

Concerning the results obtained for the cycling status of porcine bone marrow erythroid progenitors, as well as the strong Epo dependence for both

BFU-E and CFU-E (Kovačević et al., 1999), it could be concluded that the organization of the bone marrow compartment of pig erythroid progenitor cells is comparable to humans and other animals investigated. As regards the proliferative rate of peripheral blood BFU-E category of progenitor cells there are conflicting reports and more investigations need to be performed to define these values.

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OPREDELJENE MATIČNE ĆELIJE ERITROPOEZE KOSTNE SRŽI I PERIFERNE KRVI SVINJA U S FAZI ĆELIJSKOG CIKLUSA

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SADRŽAJ

Proliferativna aktivnost opredeljenih matičnih ćelija hematopoeze, odnosno procenat ovih ćelija u S (sintetskoj) fazi ćelijskog ciklusa je veoma osetljiv parametar za određivanje poremećaja fizioloških vrednosti koje nastaju dejstvom stimulatornih ili inhibitornih molekula, ili kod repopulacije hematopoeze posle mijeloablacije. Ovaj parametar je takođe vredan u ocenjivanju osetljivosti matičnih ćelija na radijacione povrede ili određene citotoksične lekove. Uprkos činjenici da su kao eksperimentalni modeli u hematopoetskim istraživanjima i u eksperimentalnoj transplantaciji kostne srži korišćeni kako visoko-srodni sojevi minijaturnih, tako i industrijske rase svinja, literaturni podaci o proliferativnoj aktivnosti opredeljenih matičnih ćelija još uvek nedostaju. Cilj ovog istraživanja je bio da se odrede proliferativne aktivnosti opredeljenih matičnih ćelija eritropoeze iz kostne srži i periferne krvi odraslih svinja u fiziološkim uslovima. Procenat opredeljenih matičnih ćelija za eritropoezu, BFU-E (Burst forming unit-erythroid) i CFU-E (Colony forming unit-erythroid) u S fazi ćelijskog ciklusa je određivan tehnikom "suicida". Metoda se zasniva na in vitro "ubijanju" ćelija u S fazi ćelijskog ciklusa citozin arabinozidom (Ara-C). Rezultati su pokazali da je procenat ranih i zrelih opredeljenih matičnih ćelija za eritropoezu u S fazi ćelijskog ciklusa različit, i iznosio je 33,3% za BFU-E i 55,1% za CFU-E ćelije. U perifernoj krvi proliferativna aktivnost BFU-E je iznosila 26,3%. Dobijeni rezultati pokazuju da je proliferativna aktivnost opredeljenih matičnih ćelija eritropoeze kod svinja slična vrednostima dobijenim kod drugih vrsta sisara.