ОГЛЯДИ

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Effect of cord blood cells on interferonogenesis and effector cells of immune system

Встановлено, що клітини кордової крові людини in vitro продукують інтерферони-α та -γ спонтанно, а також у відповідь на адекватну індукцію. Трансплантація клітин кордової крові мишам призводила до активації ендогенного інтерфероногенезу: підвищувалася концентрація сироваткового інтерферону, продукція інерферонів-α та -γ in vitro активованими лейкоцитами периферійної крові. Максимальне накопичення інтерферону у сироватці крові мишей після трансплантації клітин кордової крові не збігалося з динамікою інтерфероноутворення під впливом стандартних індукторів раннього та пізнього інтерферону – poly I:C і ридостину. Активація інтерфероногенезу супроводжувалася підвищенням функціональної активності клітин фагоцитарної системи.

INTRODUCTION

Unique biological properties of stem cells derived from embryo cells, cord blood (CB), and bone marrow show new prospects of their use in oncology, cardiology, hematology, ophthalmology, regenerative medicine etc. CB is especially important as an alternative source of mesenchymal stem cells (MSC), their use decreasing risks of graft-versus-host reaction. A high CB therapeutic effect was demonstrated in patients with secondary immunodeficiency conditions accompanying severe course of tumor and infectious diseases [1, 4, 7].

Current reports witness MSC to possess some immunomodulating properties; they function as antigen-presenting cells and product immunoregulating cytokines. MSC show also some effect on functional activity for a wide specter of immune system cells; for example, they stimulate or suppress proliferative T-lymphocyte activity and change CD4/CD8 T-cell ratio etc [11, 20]. The mechanisms of MSC stimulating or suppressing activities are found to be supported by a lot of immunoregulating cytokines including interferon-γ (IFN-γ), tumor necrosis factor a (TNFa), IL-2, and IL-10 [9]. MSC are able to develop and to differentiate to cells with mature immunocompetent phenotype synthesizing immunoregulating cytokines [19]. However, a number of problems concerning the MSC effect on the organism's cytokine profile are not yet studied in detail. In particular, it is not known how MSC change the production of endogenous IFNs; these compounds are polyfunctional biological regulators of homeostasis as well as of functional activity of immune system effector cells including also phagocytes and natural killer cells (NKC) being the main IFN target.

It must be emphasized a murine experimental model to be widely used for investigations of immune response in studying the effect of allogeneic, syngeneic, and xenogeneic MSC from CB or bone marrow [15, 17, 19]. It is demonstrated that following their transplantation the MSC migrate in recipient's organism

and appear soon in lungs, spleen, liver, intestine, and skin [18]. That is why the majority of authors think the murine experimental model to be adequate for in vivo studying of biological properties of any MSC – allogeneic, syngeneic, and xenogeneic.

Because of such situation, the aim of our investigation was to determine the CB cells ability to produce IFN in vitro as well as to understand their effect on the endogenous IFN synthesis and functional activity of immune system effector cells (phagocytes and NKC) using the murine model.

MATERIALS AND METHODS

Native human CB was taken for experiments. A fraction of nuclei-containing CB cells were isolated by accelerated "spontaneous" erythrocytes pelleting in a plasma-substituting solution, Helofusin [1]. The concentration of CB cells taken for testing was $1x10^7$ cells/ml. Surface antigens of CB lymphocytes were determined using a direct immunofluorescence approach. Monoclonal antibodies (Caltar, USA) to $CD3^+$, $CD4^+$, $CD8^+$, $CD34^+$, and $CD19^+$ antigens were used for this work. Calculation of lymphocytes as well as data analysis were made in a cytofluorimeter FACStar^{Plus} (Becton-Dickinson, USA). To investigate immunomodulating properties of CB cell non-inbred mice were taken, their body mass being 15-16 g. CB cells were introduced, once or twice, into intraperitoneal cavity or intravenously. Standard IFN inducers used were poly I:C (Sigma, USA) and ridostin (Vertor-Pharm, Russia). Control mice were treated by saline. On the 1st, 3rd, 6th, and 12th day following CB cells introduction, native peripheral blood samples were taken, and splenocytes and peritoneal macrophages were obtained according to routine approaches [5]. Cultured CB cells as well as non-fractioned murine peripheral blood cells and splenocytes were used to investigate spontaneous and induced IFN production. For IFN-γ and -α induction, a phytohemagglutinin (PHA; Difco, USA) and ridostin were used. IFN levels were titrated in an established human diploid fibroblast cell culture, M-19. The IFN activity was evaluated according to the suppression of cytopathic effect of a test virus used (vesicular stomatitis virus). Reference preparations of both IFN-γ and -α were taken for this study [5]. Functional activity of peritoneal macrophages was evaluated by routine methods determining absorbing and oxygen-depending bactericide activity. While studying the absorbing activity by a microscopic method, 100-200 cells were taken to calculate phagocytosis index (PI) – percent of cells absorbing latex particles, and also to determine phagocytic number (PN) – mean quantity of latex particles absorbed by each phagocyte. The oxygen-depending bactericide activity of macrophages was studied in a spontaneous reduction test as well as in a reduction test with pyrogenal stimulation using nitroblue tetrazolium cytochemical assay (NBT). The percent of cells (among 100 ones) was calculated containing dark-blue diformazan granules. The difference between data for spontaneous and stimulated NBT test showed the macrophage functional reserve (FR, %).

Natural killer activity (NKA) of splenocytes was assayed by a colorimetric method [9]. Index of natural killer activity (NKAI) was calculated using a formula $(To\text{-}T_c/T_o) \times 100$ (%): T_o are optical density values in experimental samples, Tc are optical density values for control samples.

All digital data obtained were analyzed using a computer program Epi Info (version 6.0) by a variation statistics approach supplemented with the Student's criterion. To evaluate individual index, their mean arithmetic values were taken ± their mean error (M±m).

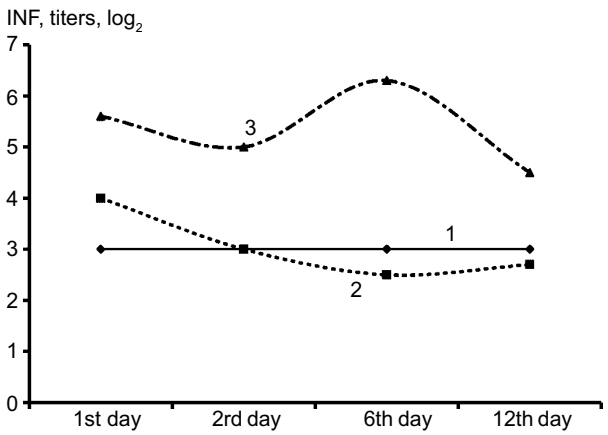
RESULTS AND DISCUSSION

Our results concerning the study of lymphocyte phenotypes show the CB to contain $CD3^+$ T-lymphocytes (56.7±5.6 %), $CD4^+$ T-help-

ers/inducers (37.3±8.1 %), $CD8^+$ T-suppressors/cytotoxic lymphocytes (29.5±6.5 %), $CD19^+$ B-lymphocytes (13.7±4.2 %), and hemopoietic precursor cells expressing CD34+ antigen (1.8±0.6 %).

Non-fractioned CB cells were proved to possess an IFN-inducing activity. In vitro these cells induced this cytokine spontaneously, its titer reaching 3.10±0.02 $\log_2$ conventional U per ml (CU/ml). Following induction by the PHA or ridostin the titers reaching 4.80±0.02 $\log_2$ CU/ml and 3.60±0.02 $\log_2$ CU/ml for IFN-γ and IFN-α, respectively. It should be emphasized the spontaneous IFN level produced by cultivated CB cells was almost twice higher comparing to IFN levels synthesized by peripheral blood leucocytes taken from clinically healthy young children [3, 6], IFN-γ titers being twice higher. Interestingly, stimulated CB cells produced more actively the IFN-g comparing to the IFN-α; such fact may be due to high percent of T-lymphocytes belonging to the Th1-type witch produce the IFN-γ.

It was also shown using the experimental murine model, xenogeneic CB cells to influence on endogenous IFN synthesis as well as on functional activity of immune system effector cells. The serum IFN levels were demonstrated to be changed in graft-carrying mice, the changes being dependent on the mode of CB cells introduction (Figure). A significant IFN accumulation was a result of intravenous CB cells injection. Following a single CB cells introduction, a slight increase of serum IFN titers was seen on the 1st and 3rd days (5.60± 1.30 and 5.00±1.20 $\log_2$ CU/ml, respectively), the control level being 3.00±0.20 $\log_2$ CU/ml ($p > 0,05$). On the 6th day a significant serum IFN accumulation was seen – up to 6.30±0.09 $\log_2$ CU/ml ($p < 0,05$), its level decreasing up to control values on the 12th day (4.50±1.00 $\log_2$ CU/ml; $p > 0,05$). On the same time, injections of standard IFN inducers – poly I:C or ridostin – caused the IFN level increase in 6 h (7.00±1.00 $\log_2$ CU/ml; $p < 0,05$) and on the 3rd day (6.80±0.90 $\log_2$ CU/ml; $p < 0,05$) respectively. Following two CB cells injections the serum IFN levels became significantly higher on the 3rd day already (12.40±1.00 $\log_2$ CU/ml, $p < 0,05$), IFN titers becoming almost twice higher comparing to mice treated only once. The serum IFN level kept increased up to the 6th day (5.00±0.03 $\log_2$ CU/ml, $p < 0,05$) reaching gradually control levels on the 12th day (4.00±0.90 $\log_2$ CU/ml, $p > 0,05$). A single intraperitoneal injection of CB cells caused a slight increase of murine serum IFN on the 1st day, the difference between treated and control animals being, however, not statistically significant (4.00±0.60 $\log_2$ CU/ml for treated animals and 3.00±0.20 $\log_2$ CU/ml for control ones; $p > 0,05$). The IFN concentration in blood sera kept on control levels on the 3rd, 6th, and 12th days (3.00±0.10, 2.50±0.90, and



Changes of serum interferon levels in intact mice following single introduction of cord blood cells: 1 – intact mice, 2 – intraperitoneal injection, 3 – intravenous injection

2.70±0.30 $\log_2$ CU/ml, respectively).

Murine peripheral blood leucocytes were demonstrated to be able to begin the IFN synthesis in vitro following the introduction of the CB in vivo. Such spontaneous IFN production by peripheral blood leucocytes did not change following both single intraperitoneal and single intravenous CB injection (Table 1). However, peripheral blood leucocytes taken from animals following intravenous CB injection produced higher IFN-α and IFN-γ quantities after induction by PHA or ridostin comparing to control leucocytes. On the 3rd and 6th days following intravenous CB injection, IFN-α and -γ titers became higher, serum IFN levels reaching then their maximal levels. As it is seen in the Table 1, intraperitoneal injection of CB cells did not change in vitro IFN-α and -γ synthesis by activated murine peripheral blood leucocytes. It should be emphasized no changes of IFN-inducing activity were found for splenocytes. IFN titers produced by splenocytes both spontaneously and following adequate induction kept on their control levels (Table 2).

It goes without saying intravenous CB injection to cause a prolonged activation of endogenous IFN synthesis proved by increased serum IFN levels and ability of murine peripheral blood leucocytes to synthesize IFN-α and -γ in vitro following an adequate induction.

Our results prove the activation of endogenous CB-stimulated IFN synthesis to be accompanied by increased functional activity of peritoneal macrophages. The table 3 shows FI and FN values did not change comparing to the control ones on the 1st day following CB injection; however, they became significantly higher on the 3rd, 6th and 12th day. The intraperitoneal CB injection led to FI increase on the 3rd and 6th day, this index becoming gradually lower up to the control level on the 12th day. At the same time, the phagocytosis activity was observed to increase only on the 3rd day. The metabolic activity of macrophages from CB-treated mice demonstrated a sharp increase of the oxygen-dependent bactericide activity (Table 2). A quantity of NTB-positive macrophages in a spontaneous test rose following intravenous CB cells administration during the whole time of observation – up to 12 days, such increase being seen only during a day following intraperitoneal injection. At the same time the oxygen-dependent macrophage activity induced by pyrogenal stimulation did not increase markedly both following intravenous and intraperitoneal injection of CB cells, the FR being below control levels. It may be due to ex-

Table 1. Interferon production by murine peripheral cells following cord blood cells injection

Groups of mice observed /time of observation		Interferon titers, $\log_2$ CU/ml		
		Spontaneous	Interferon-γ	Interferon-α
Control		3.40±0.10	3.30±0.08	3.00±0.02
Single intraperitoneal introduction of cord blood cells				
Obtaining of CB cells	1st day	2.50±0.20	2.30±0.10	3.20±0.04
	3rd day	4.00±0.08	3.00±0.07	3.00±0.10
	6th day	4.00±0.10	4.00±0,12	4.00±0.08
	12 day	4.00±0.11	3.80±0.05	3.10±0.90
Single intravenous introduction of cord blood cells				
Obtaining of CB cells	1st day	3.30±0.09	3.33±0.03	3.30±0.03
	3rd day	4.50±0.12	5.00±0.10*	5.30±0.01*
	6th day	4.000.21	4.50±0.02*	4.54±0.09*
	12th day	3.60±0.07	3.90±0.04	3.01±0.14

* $p < 0,05$ relatively to control data

hausted natural reserve possibilities of excessively activated macrophages following CB cells administration. It should be noted the peak macrophages activation to coincide with the maximal serum IFN accumulation and increased production of IFN-α and -γ by peripheral blood cells.

At the same time, the activity of murine splenic NKC did not change following intravenous CB cells introduction. On the 1st day the index of NKA was 32.2±2.3 conv.U comparing to 30.5±3.2 conv.U in control, a tendency of this value decrease being found on the 3rd day (24.4±5.1 conv.U; $p > 0,05$). On the 6th and 23th days the NKA index did not exceed its control level (29.4±3.2 and 27.8±4.6 conv. units, respectively).

Thus, our results show that CB cells produce the IFN in vitro; these cells administrated intravenously to intact mice activated both endogenous IFN synthesis and function of murine phagocytic system. The production of IFN -α and IFN-γ in vitro is associated with the presence of cells synthesizing these cytokines among CB, in particular of $CD4^+$ T-helpers/inducers and $CD19^+$ B-lymphocytes. Interestingly, MSC may produce IFN spontaneously, especially the IFN-γ participating in a mechanism of their immunomodulating function stimulating and suppressing the proliferative T-lymphocyte activity [10, 13, 16]. Besides, there are publications demonstrating the ability of CB cells to influence on the production of IFNs and other immunoregulating cytokines in vitro in mixed cultured lymphocyte cells. The MSC were found to induce the IFN-γ and IL-10 synthesis in lymphocytes, the IL-4 and TNF-α production becoming suppressed [8, 14]. That is why it is possible the stimulation of endogenous IFN synthesis in graft-carrying mice to be due to direct IFN production by CB cells in vivo and/or to CB cells caused induction of such production in recipient cells. Of interest, the increased serum IFN titers following two CB cells injections were seen earlier comparing to the single administration.

It cannot be also excluded xenogeneic human CB stem cells differentiate in murine organism following transplantation becoming immunocompetent cells able to produce IFNs and other immunoregulating cytokines. It is proved indirectly by a fact the peak of serum IFN accumulation to be seen on the 6th day following intravenous injection of CB cells. It means the IFN synthesis to occur de novo, the intraperitoneal CB injection having no effect on the serum IFN production during 12 days. It should be noted the increased serum

Table 2. Interferon production by murine spleen cells following cord blood cells injection

Groups of mice observed /time of observation		Interferon titers, $\log_2$ CU/ml		
		Spontaneous	Interferon-γ	Interferon-α
Control		2.60±0.09	2.90±0.11	3.30±0.12
Single intraperitoneal introduction of cord blood cells				
Obtaining of CB cells	1st day	2.00±0.10	3.30±0.09	3.01±0.09
	3rd day	2.10±0.09	2.00±0.10	3.00±0.20
	6th day	2.50±0.10	2.00±0.07	4.00±0.10
	12th day	4.000.20	2.12±0.06	1.50±0.09
Single intravenous introduction of cord blood cells				
Obtaining of CB cells	1st day	2.70±0.11	3.30±0.13	4.00±0.10
	3rd day	3.00±0.02	3.30±0.08	4.00±0.09
	6th day	3.300.10	2.60±0.07	3.00±0.11
	12th day	2.07±0.08	3.00±0.14	2.01±0.09

IFN level in animals treated once by intravenous CB injection to have no coincidence with IFN production dynamics following introduction of standard early and late IFN inducers – poly I:C and ridostin, respectively. Such hypothesis is supported by a recently described possibility of in vivo differentiation for hemopoietic murine CB stem cells, the last becoming mature $CD4^+$ and $CD8^+$ T-lymphocytes; the same is also found for B-cells following their syngeneic transplantation to mice. T-lymphocytes recognize specifically antigens together with major histocompatibility complex molecules and become activated. Following their stimulation by antigens, the $CD4^+$ T-lymphocytes produce cytokines including also the IFN-γ; the $CD8^+$ T-lymphocytes stimulated in mixed cultured lymphocyte cells realize a specific lysis of target cells [19]. Moreover, the human $CD34^+$ cells obtained by CB cells sorting using a MACS approach cause the increase of T-lymphocyte subpopulation expressing TCR Vbeta11 receptors in peripheral blood, bone marrow, and spleen [9]. However hemopoietic stem cells can get accustomed to the recipients organism and they can be differentiated to mature immunocompetent cells under two conditions. First, in the presence of necessary microenvironment and in the absence of competition with host cells (in the syngeneic system it could be achieved by irradiation in order to exhaust an endogenic pool of stem cells). Second, in the absence of immune uncompatibility after xenogeneic and halogeneic transplantation. For solving this problem some additional studies should be performed.

The data obtained lead to a question whether CB cells keep their viability following xenogeneic transplantation and whether these cells are able to be differentiated to cytokine-producing immunocompetent cells of mature phenotype. Recently, an answer has been found due to special studies [20]. Human CB stem cells cultivated during a long time in vitro or human stem CB cells transfected by human GM-CSF-/SCF-carrying genes were injected into murine tail vein. In 6-8 weeks a possibility of human MSC differentiation to murine hemopoietic cells was studied using a method of flow cytofluorimetry and fluorescent hybridization in situ. Human CB MSC were demonstrated to participate in murine hemopoiesis following xenogeneic transplantation to mice. However, the differentiation potential was higher in MSC transfected by genes of hemopoietic growth factors influencing on lymphocyte migration and adhesion. At the same time, the expediency of murine

Table 3. Functional activity of murine peritoneal macrophage activity following cord blood cells injection to intact mice

Groups of mice observed /time of observation		PI, %	PN,(CU)	NTB spont., %	NTB stim.,%	FR, %
Control		30.6±3.2	3.2±0.9	16.6±2.1	26.5±3.2	9.9±1.1
Single intraperitoneal introduction of cord blood cells						
Obtaining of CB cells	1st day	38.8±2.5	2.2±1.0	72.9±3.4*	74.2±4.2*	1.3±0.5*
	3rd day	41.6±4.6*	4.8±0.5*	23.5±2.8	24.5±3.4	1.0±0.1*
	6th day	55.6±2.1*	3.4±1.1	38.2±2.9	41.0±2.3	2.8±0.2*
	12th day	32.8±5.1	2.9±0.9	15.1±3.2	19.3±2.0	4.2±0.6*
Single intravenous introduction of cord blood cells						
Obtaining of CB cells	1st day	34.6±5.1	3.9±0.8	64.4±4.5*	68.3±4.1*	3.9±0.5*
	3rd day	57.3±2.5*	5.0±0.9*	73.5±5.1*	69.4±3.6*	1.0±0.1*
	6th day	46.2±2.1*	4.1±0.3*	71.1±3.9*	67.8±3.1*	1.7±0.2*
	12th day	47.8±1.9*	4.1±0.5*	42.6±2.3*	43.5±2.2*	1.0±0.2*

* $p < 0,05$ comparing to control data

xenogeneic model use for investigations of MSC biological properties, especially of MSC derived from rat bone marrow, was questioned by some researchers. It was due, first of all, to a fact that rat bone marrow MSC keeping their viability even in 11 weeks following their injection to mice cause the development of specific immune response of cytotoxic T-lymphocytes. In particular, increased proliferative and cytotoxic activities of murine specific cytotoxic T-lymphocytes were detected preventing the differentiation of rat bone marrow cells to osteoclasts [23]. It should be noted an immune response of the type graft-versus-host reaction to develop oftener following bone marrow MSC injection comparing to CB MSC [1, 4]. Of interest, we have found a tendency to decreased NKC activity following CB cells transplantation to mice, our data being agreed with results of other researchers [13]. It promotes the human cell survival following CB injection to mice, activated NKC being known to carry out the lysis of MSC [21].

Thus, our results suggest the CB cells to possess immunomodulating properties. On the one hand, they produce IFNs in vitro and stimulate in vivo IFN synthesis detected due to increased serum IFN levels and to ability of peripheral blood leucocytes to synthesize the IFN-α and -γ. Besides that, a marked activation of phagocytic system cells is seen to be able to stimulate the organism's resistance against infections. It gives a motive for further investigations of CB cells activity against infective agents of both bacterial and viral origin causing the establishment of secondary immunodeficiency conditions.

CONCLUSIONS

1. Human CB cells are able to produce in vitro both IFN-α and -γ spontaneously or following adequate stimulation.

2. CB cells injected intravenously activated endogenous IFN synthesis proved by the stimulation of IFN-α and -γ production by peripheral blood leucocytes following an adequate induction, the serum IFN levels becoming higher.

3. IFN accumulation in blood serum following CB transplantation did not coincide with IFN synthesis dynamics induced by standard early and late IFN-inducers – poly I:C and ridostin.

4. Activation of endogenous IFN synthesis due to the CB injection was accompanied by increased activity of phagocytic system function suggesting the promotion of bactericide potency of murine peritoneal macrophages due to its phagocytic and oxygen-dependent components.

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EFFECT OF CORD BLOOD CELLS ON INTERFERONOGENESIS AND EFFECTOR CELLS OF IMMUNE SYSTEM

Human cord blood cells were shown to produce interferon-α- and -γ *in vitro*, both spontaneously and following adequate induction. Transplantation of human blood cord cells to mice led to endogenous interferonogenesis activation, serum interferon levels as well as interferon-α and interferon-γ synthesis by activated peripheral blood leucocytes becoming higher. The maximal interferon accumulation in murine blood sera following transplantation did not coincide with interferonogenesis dynamics observed due to the effect of standard early and late interferon inducers – poly I:C and ridostin, respectively. Interferonogenesis activation was accompanied by increased functional activty of phagocytic system cells.

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