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FROZEN-DRIED HUMAN CORD BLOOD LEUKOCONCENTRATE AS A DRIVER OF HSP70 PROTEIN EXPRESSION AND TOLEROGENIC DENDRITIC CELLS FORMATION

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Dendritic cells play a leading role in regulating the immune response, particularly in the induction of immune tolerance. The heat shock protein Hsp70 is known for its immunomodulatory properties. To date, its participation in the formation of the tolerogenic phenotype and function of dendritic cells remains poorly understood. The effect of frozen-dried human cord blood leukoconcentrate on the expression of Hsp70 protein in dendritic cells and the associated changes in their functional state was analyzed. The dose-dependent ability of frozen-dried human cord blood leukoconcentrate to induce Hsp70 expression in dendritic cells derived from mouse bone marrow mononuclear cells was established, indicating its regulatory potential. It has been shown that, under the influence of frozen-dried human cord blood leukoconcentrate, the expression of the Hsp70 protein in dendritic cells increases, thereby enhancing their tolerogenic function by increasing the production of the anti-inflammatory cytokine IL-10. The results emphasize the key role of the Hsp70 protein in inducing immune tolerance and also open up prospects for the use of frozen-dried human cord blood leukoconcentrate in the treatment of autoimmune pathologies.

Key words: tolerogenic dendritic cells, frozen-dried human cord blood leukoconcentrate, Hsp70 protein, IL-10.

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ЛІОФІЛІЗОВАНИЙ ЛЕЙКОКОНЦЕНТРАТ КОРДОВОЇ КРОВІ ЛЮДИНИ ЯК ДРАЙВЕР РІВНЯ ЕКСПРЕСІЇ БІЛКА HSP70 І ФОРМУВАННЯ ТОЛЕРОГЕННИХ ДЕНДРИТНИХ КЛІТИН

Провідну роль у регуляції імунної відповіді, зокрема у формуванні імунної толерантності, відіграють дендритні клітини. Білок теплового шоку Hsp70 відомий своїми імуномодулюючими властивостями. До теперішнього часу його участь у формуванні толерогенного фенотипу і функції дендритних клітин залишається недостатньо вивченою. Проаналізовано вплив ліофілізованого лейкоконцентрату кордової крові людини на експресію білка Hsp70 в дендритних клітинах та пов'язані з цим зміни їх функціонального стану. Встановлена дозозалежна здатність ліофілізованого лейкоконцентрату кордової крові на експресію білка Hsp70 в дендритних клітинах, отриманих з мононуклеарів кісткового мозку мишей, що свідчить про його регуляторний потенціал. Показано, що під впливом ліофілізованого лейкоконцентрату кордової крові в дендритних клітинах підвищується експресія Hsp70 білка, що визначає їх толерогенну функцію за збільшенням продукції протизапального цитокіну ІЛ-10. Отримані результати акцентують увагу відносно ключової ролі білка Hsp70 в індукції імунної толерантності, а також відкривають перспективи використання ліофілізованого лейкоконцентрату кордової крові у терапії патологій аутоімунного генезу.

Ключові слова: толерогенні дендритні клітини, ліофілізований лейкоконцентрат кордової крові, білок Hsp70, ІЛ-10.

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Dendritic cells (DCs) are increasingly being considered as potential correctors of the immune response in various pathological conditions [2, 13]. Indeed, by supporting T-cell tolerance, tolerogenic DCs (tolDCs) have been applied in the therapy of autoimmune pathologies of various etiologies. TolDCs perform their function in vivo in various ways, namely by inducing anergy, deleting antigen-reactive T cells, stimulating T regulatory cells with suppressor function, and producing anti-inflammatory cytokines, in particular interleukin-10 (IL-10) [3, 7, 11]. Currently, various approaches exist for generating tolDCs ex vivo, differing in their suitability for multicomponent agents with broad functional effects. Recently, attention has been drawn to the unique properties of cord blood, particularly its capacity to induce tolerance. These data open the prospect of using human cord blood (HCB) as an alternative source with immunomodulatory properties to restore the body's immunity, particularly by increasing the tolerogenic potential of DCs [12]. Given this, it is advisable to use frozen-dried human cord blood leukoconcentrate (fdHCBL) as an inducer of tolDCs formation for clinical purposes. An additional prerequisite for its use as a tolerance-inducing factor, along with other biologically active compounds, is the presence of IL-10 in plasma [8]. Indeed, IL-10 is used to induce tolDCs alongside immunosuppressants, anti-inflammatory cytokines, or genetic modifications [1, 5]. IL-10 modulates the differentiation and function of myeloid cells, leading to the formation of tolDCs with the most potent tolerogenic characteristics.

Improvements in methods for generating tolDCs using IL-10 [4] have shown that different types of tolDCs can be obtained depending on whether IL-10 is added at the beginning of cultivation or only at the final stage. In particular, the authors

indicate that when monocytes are cultivated in the presence of GM-CSF and IL-4 with the addition of IL-10 at the beginning of the experiment, DCs are formed, designated as DC-10, positive for the CD14 marker with a high level of expression of the CD83, CD80, and CD86 molecules, which is inherent in mature DCs. Such DC-10, unlike mature DCs, produced IL-10 and exhibited a specific function of inducing Tr1 cells. When using a different protocol that included IL-10 at the final stage of cultivation, DCs were obtained with an immature phenotype. They demonstrated resistance to maturation stimuli, leading to the formation of anergic $CD4^+$ T cells [9].

It is known that the synthesis of an anti-inflammatory cytokine such as IL-10 is under the control of heat shock proteins of the Hsp70 family [14, 15]. The authors point to the ambiguous role of Hsp70 in immune regulation, underscoring the need for further study. It has been noted that intracellular Hsp70 proteins are activators of the anti-inflammatory process [14, 15]. The immunosuppressive effect of the aforementioned proteins is mediated by inhibition of nuclear factor kappa B (NF-kB) activity, thereby modulating the tolerogenic phenotype and DC functions, with increased IL-10 synthesis. Interestingly, the opposite effect occurs when Hsp70 is extracellular. It is suggested that extracellular Hsp70 may act as a damage-associated molecular pattern (DAMP) via TLR2 and TLR4, thereby stimulating an immune response that leads to inflammation. This fact is associated with increased NF-kB expression, which has been observed in response to extracellular Hsp70 in human lung cancer [14].

Therefore, Hsp70 proteins play a multifaceted and very important role, particularly in the induction of tolerance in DCs. In this regard, an appropriate indicator of the state of DCs obtained from mononuclear cells (MNCs) of the bone marrow of animals in the presence of fdHCBL is the assessment of Hsp70 protein content. In general, the disclosure of the molecular mechanisms underlying the increase in the tolerogenic potential of DCs, mediated by the expression of the Hsp70 protein under the influence of fdHCBL, may contribute to the development and improvement of approaches to treating diseases of autoimmune origin.

The purpose of the study was to determine the effect of frozen-dried human cord blood leukoconcentrate on the nature of the formation of the tolerogenic potential of dendritic cells obtained from animal bone marrow mononuclear cells.

Materials and methods. The experiments were performed on CBA/H mice in accordance with the Law of Ukraine "On the Protection of Animals from Cruelty" (No. 3447–IV dated 21.02.2006).

The experiments were performed on 6-month-old female CBA/H mice, which were kept under standard vivarium conditions of the Institute of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. The surveys were conducted between April and June 2025. All manipulations with animals were approved by the Bioethics Committee of the Institute of Cryobiology and Cryomedicine of the NAS of Ukraine (Protocol No. 5 dated 11/26/2019) and comply with the main provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and the Law of Ukraine "On the Protection of Animals from Cruelty". All experiments, biological material processing, and data analysis were carried out at the Institute of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. The study used 20 mice to obtain bone marrow mononuclear cells, which were subsequently cultivated.

To obtain a suspension of bone marrow cells, intact mice were euthanized by decapitation. Femurs were removed, epiphyses were cut off, and bone marrow was washed out of the femurs of mice using a syringe with RPMI-1640 medium (Biowest, France) supplemented with 3 % fetal bovine serum (Biowest, France) and 2 % sodium citrate (Sigma–Aldrich, USA) (hereinafter referred to as the culture medium). The bone marrow cell suspension was filtered through a 100 μm pore size filter (Falcon, USA), centrifuged (Digicen 20-R, Los Frailes, Spain) at 200g for 10 min, and the cell pellet was resuspended in culture medium.

Isolation of MNCs from bone marrow cell suspension was carried out by centrifugation in a density gradient (1.077 g/ml) of the drug "Trazograf 76 %" (Unique Pharmaceutical Laboratories, India) [6].

The MNCs obtained in this way were cultivated in Petri dishes (d=3 cm) at a concentration of 3–5×10^6 cells/mL immediately after their isolation in RPMI-1640 medium supplemented with 10 % fetal bovine serum. Cells were cultured at 37°C in a 5 % CO_2 atmosphere. After 2 hours, the medium with non-adherent cells was removed, and RPMI-1640 culture medium containing mouse recombinant GM-CSF, IL-4, and dexamethasone (Sigma-Aldrich, USA) (basal medium) was added to monocytes (adhesive fraction of MNCs) [6]. After 4 days, a fresh portion of the basal medium with fdHCBL was added. This time point of fdHCBL addition, when the plasma contains IL-10, was chosen for the final stage of cultivation, in accordance with protocol [9], which enables the production of DCs with tolerogenic potential and resistance to maturation. On Day 7, cells obtained from MNCs were collected for further cytofluorimetric analysis of their affiliation with tolDCs.

Human cord blood leukoconcentrate (HCBL) in autologous plasma was obtained by sedimentation of red blood cells after the addition of Polyglucinum to cord blood (Yuria LLC–Pharm, Ukraine) and was freeze-dried according to the method of A.M. Goltsev et al. (Patent No. 113006, 2017). Samples of fdHCBL stored at 4°C. Rehydration of fdHCBL was carried out by adding 1 ml of physiological solution to the vials ("Yuria-Pharm", Ukraine). Rehydrated fdHCBL in various doses: $1x10^3$, $1x10^4$, and $1x10^5$ cells/mL were added to the basal MNC cultivation medium on Day 4.

The following groups were formed, n=5 in each: Group 1 – Control group (without addition of fdHCBL); Group 2 – with the addition of $1x10^3$ cells/mL of fdHCBL; Group 3 – with the addition of $1x10^4$ cells/mL of fdHCBL; Group 4 – with the addition of $1x10^5$ cells/mL of fdHCBL. Three independent experiments were conducted.

The content of the heat shock protein Hsp70 was determined by the flow cytometry method on a FACS Calibur Flow Cytometer (Becton Dickinson, USA) using FITC-conjugated anti-mouse Hsp70 monoclonal antibodies (Abcam, United Kingdom). As an additional indicator for assessing the expression of membrane markers on immunocompetent cells obtained from MNCs, we used mean fluorescence intensity (MFI), which reflects the density of the corresponding marker on the cell. The total light index (TLI) was also determined, an integral indicator of marker expression. The total light index was calculated as the product of the number of cells expressing the corresponding marker per MFI.

IL-10 content was determined on Day 7 in DC culture supernatants using the Cytometric Bead Array (CBA) Th1/Th2/Th17 (mouse) Kit IL-10 set (BD Biosciences, USA) according to the manufacturer's instructions.

Statistical data processing was carried out using Microsoft Excel 2010 and Statistica 10.0 (StatSoft, USA). Quantitative data were processed using the method of variational statistics, with calculation of the median and interquartile range (Me [25; 75]). Comparison of independent samples was performed using the Mann-Whitney method. Differences were considered statistically significant at $p<0.05$.

Results of the study and their discussion. As noted above, Hsp70 proteins play an important role in inducing the tolerogenic potential of DCs. At the same time, assessing the degree of Hsp70 protein expression in DCs indicates the manifestation of its tolerogenic function. Table 1 presents data indicating an increase in the number of $Hsp70^+$ DCs derived from MNCs when fdHCBL are added to the culture medium.

Table 1

Features of Hsp70 protein expression in DCs obtained in vitro from MNCs in the presence of different doses of fdHCBL, Me [25; 75], n=5

Group No.	DC	Number of $Hsp70^+$DC, %	MFI, CU	TLI, CU
1	Control (without fdHCBL)	8.9 (8.4; 9.6)	2698.3 (2675.4; 2715.1)	24164.4 (22880.8; 25847.5)
2	$+1x10^3$ cell/mL of fdHCBL	11.8 (10.8; 12.2) *	2912.7 (2876.3; 3002.5) *	33723.2 (31455.0; 36046.6)*
3	$+1x10^4$ cell/mL of fdHCBL	15.5 (14.8; 15.9)*	2448.5 (2385.2; 2469.4)*	36794.0` (35301.0; 38459.6)*
4	$+1x10^5$ cell/mL of fdHCBL	14.3 (13.9; 14.3)*	2104.2 (2076.7; 2156.7)*	30090.6 (29330.4; 30840.8)*

Note: MFI – mean fluorescence intensity; TLI – total light index; * – indicators are significantly different from the Control ($p<0.05$ by Mann-Whitney U-test).

As shown in the data, all doses of fdHCBL increased the number of $Hsp70^+$DCs compared with the control (Group 1). At the same time, a clear dose-dependent effect of the influence of fdHCBL on the formation of $Hsp70^+$ DCs was observed. The maximum increase in the number of $Hsp70^+$DCs by 1.7 times was observed when adding fdHCBL at a dose of 1×10^4 cells/mL (Group 3). The ability to identify the Hsp70 protein by MFI per cell increased significantly upon adding the lowest dose of fdHCBL (1×10^3 cells/mL; Group 2), compared to the control (2912.7 and 2698.3 CU, respectively). However, the integral expression index of Hsp70 protein in DCs was maximal (36794.0 conditional units) under the conditions of adding fdHCBL at a dose of 1×10^4 cells/mL (Group 3), which was 1.5 times higher than the corresponding index in the control (24164.4 CU). At the same time, increasing the dose of fdHCBL to 1×10^5 cells/mL (Group 4) was not accompanied by a further increase in either the number of $Hsp70^+$ DCs or the level of Hsp70 protein expression in them, considering by the MFI and TLI indices.

The results of assessing IL-10 content in supernatants from DC cultures in vitro derived from MNCs in the presence of different doses of fdHCBL confirm its dose-dependent tolerance-inducing ability (Fig. 1). Thus, when adding leukoconcentrate to the basal culture medium at doses of $1x10^3$ cells/mL and $1x10^4$ cells/mL, an increase in IL-10 DC production was observed, with a maximum at

$1x10^4$ cells/mL. Under these conditions, IL-10 production in DC culture supernatants increased 1.4-fold compared with the control (10.4 pg/mL vs. 7.4 pg/mL).

This finding may indicate that fdHCBL, when used at a dose of $1x10^4$ cells/mL, can activate tolDC production in vitro, as indicated by the maximum IL-10 production. Increasing the dose of fdHCBL to $1x10^5$ cells/mL, on the contrary, led to a 20 % decrease in the concentration of IL-10 in DC culture supernatants compared to the control (16.0 pg/mL and 7.4 pg/mL, respectively).

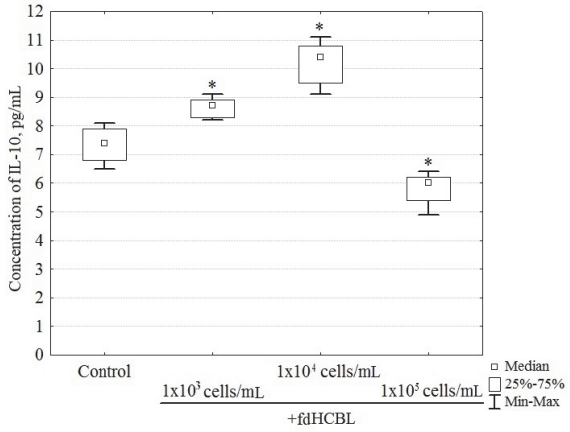

Fig. 1. IL-10 concentration in DC culture supernatants obtained in vitro from MNCs in the presence of different doses of fdHCBL.

Note: * – the indicator has statistically significant differences compared to the control ($p < 0.05$, according to the Mann-Whitney U-criterion).

HCB components are a rich source of cells, cytokines, and other factors that have anti-inflammatory and immunosuppressive effects [12]. In our previous studies [10], it was found that when different concentrations of leukoconcentrates ($1x10^3$, $1x10^4$, $1x10^5$ cells/mL of culture medium) were added to the bone marrow MNC culture, a dose-dependent effect of leukoconcentrate in regulating both the phenotype and functional state of DCs was demonstrated. Frozen-dried human cord blood leukoconcentrate at a dose of $1x10^4$ cells/mL maximally increased the number of $CD11b^+$ DCs and the level of expression of the corresponding molecule on the membrane, while reducing the number of $CD80^+$ and $CD86^+$ cells and the level of expression of these costimulatory molecules, which confirms their tolerogenic function. An important role in the implementation of the tolerogenic function of DCs is also played by the intracellular protein Hsp70, which activates the anti-inflammatory process [14, 15]. Our studies showed that the maximum increase in the number of $Hsp70^+$DCs and the level of this protein's expression, relative to the TLI index, was observed when fdHCBL was added at a dose of 1×10^4 cells/mL in the culture medium. It should be noted that increasing the dose of fdHCBL to 1×10^5 cells/mL was not accompanied by a further increase in the number of $Hsp70^+$ DCs and the level of Hsp70 protein expression in them. That is, this indicates the presence of protein components in the composition of fdHCBL that regulate the tolerogenic activity of DCs, achieved through the production of the anti-inflammatory IL-10 [14].

It is known that the synthesis of such an anti-inflammatory cytokine as IL-10 is under the control of heat shock proteins of the Hsp70 family [15]. Indeed, the results show that when adding $1x10^4$ cells/mL of fdHCBL to the basal culture medium, a maximum increase in IL-10 production by dendritic cells was observed by 1.4 times compared to the control. It is significant that under these cultivation conditions, an increase in both the percentage of $Hsp70^+$ DCs (by 1.7-fold) and the TLI index for this protein (by 1.5-fold) was observed, consistent with the control values. These data confirm the mediated nature of the interdependence between Hsp70 protein expression in DCs and their production of IL-10.

Conclusions

1. The dose-dependent ability of frozen-dried human cord blood leukoconcentrate to induce Hsp70 protein expression in dendritic cells derived from animal bone marrow mononuclear cells was established. This indicates its potential to regulate the tolerogenic activity of these cells.

2. The optimal concentration of frozen-dried human cord blood leukoconcentrate ($1x10^4$ cells/mL) was determined, which maximally stimulates the tolerance-inducing ability of $Hsp70^+$ dendritic cells obtained from bone marrow mononuclear cells by the level of expression of this protein, which was 1.5 times higher than the corresponding indicator in the control. Increasing the dose of frozen-dried human cord blood leukoconcentrate to $1x10^5$ cells/mL was not accompanied by a further increase in both the number of $Hsp70^+$ dendritic cells and the level of Hsp70 protein expression in them.

3. The maximum enhancement of IL-10 production in the supernatants of dendritic cell cultures was established after the addition of frozen-dried human cord blood leukoconcentrate at a concentration of $1x10^4$ cells/mL to the basal culture medium, which confirms the indirect nature of the interdependence between the expression of the Hsp70 protein in dendritic cells and their production of IL-10.

Disclosure of the molecular mechanisms underlying the increase in the tolerogenic potential of dendritic cells, mediated by Hsp70 expression induced by frozen-dried human cord blood leukoconcentrate, may contribute to the development and improvement of approaches to treating autoimmune diseases.

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Conflict of interest. The authors have no conflicts of interest to declare.

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