ВЛИЯНИЕ СУПЕРНАТАНТА *BIFIDOBACTERIUM BIFIDUM* НА МОРФОФУНКЦИОНАЛЬНЫЕ СВОЙСТВА ФИБРОБЛАСТОВ ЧЕЛОВЕКА В ДИНАМИКЕ В ЭКСПЕРИМЕНТЕ *IN VITRO*

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Резюме. В настоящее время активно идет поиск экзогенных стимуляторов процессов репарации и регенерации. В последние десятилетия накоплены данные об иммунотропной активности бифидобактерий. Ключевая же роль в восстановлении дефектов в области раны принадлежит фибробластам за счет секреции компонентов внеклеточного матрикса, метаболитов, сигнальных факторов для окружающих клеток и регуляции тканевого метаболизма. В работе приведены результаты исследования влияния супернатанта *Bifidobacterium bifidum* (10 мкл/мл) на морфофункциональные свойства фибробластов человека в динамике в эксперименте *in vitro*. В работе использовали эталонный штамм *Bifidobacterium bifidum* 791 (Всероссийская коллекция промышленных микроорганизмов ФГУП ГосНИИ «Генетика», № депонента АС-1247), использующийся при производстве пробиотика «Бифидумбактерин» (ЗАО «Экополис», г. Ковров) и фибробласты взрослого человека (линия клеток ЛЭЧ-4(81)) (лаборатория клеточных культур ЕНИИВИ, г. Екатеринбург). Структурно-функциональные исследования проводили на 1-е, 3-и, 7-е, 14-е, 21-е, 28-е сутки сокультивирования. Продукты вторичного метаболизма B. bifidum оказывают стрессовое воздействие на морфофункциональное состояние фибробластов в первые сутки. Стимулируют процессы пролиферации в культуре в опыте 2,67±0,24 в сравнении с контролем 0,75±0,15 ($p < 0,01$), не блокируя при этом апоптоз в клетке. Это приводит к усилению продукции белков внеклеточного матрикса, как коллагена (пг/мл) (400±19 против 110±25 в контроле), так и эластина (нг/мл) 395±30 и 125±29). Сокультивирование фибробластов в опытном образце в течение суток приводит к массивному «сбрасыванию» рецептора CD44 ($p < 0,05$), в отличие от контроля, которое подтверждается фенотипическими изменениями ($r = 0,66$). На 1 и 3 сутки наблюдается снижение $CD105^+$, $CD44^+$ рецепторов ($p < 0,05$), по сравнению с контрольной группой и увеличение экспрессии $CD29^+$ ($p < 0,05$). Активированные фибробласты обладают измененным секреторным фенотипом, продуцирующим цитокины различной направленности TGF-β ($r = 0,78$), IL-6 ($r = 0,57$), IL-1β ($r = 0,75$), IL-8 ($r = 0,63$). Максимальная адаптация клеток в опытной систе-

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ме регистрируется на 7-е сутки, что коррелирует с морфометрическим (r = 0,59) и цитометрическим (r = 0,71) исследованиями. Полученные данные способствуют пониманию механизмов иммунорегуляторного влияния нормобиоты (на модели бифидобактерий) на процессы репарации и регенерации.

Ключевые слова: фибробласты, Bifidobacterium bifidum, цитокины, коллаген, эластин, проточная цитометрия

EFFECT OF *BIFIDOBACTERIUM BIFIDUM* SUPERNATANT ON THE MORPHOLOGICAL AND FUNCTIONAL CHARACTERISTICS OF HUMAN FIBROBLASTS IN REAL TIME DURING AN *IN VITRO* EXPERIMENT

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Abstract. Currently, there is an active search for exogenous stimulators of repair and regeneration processes. In the recent decades, some data on the immunotropic activity of bifidobacteria have been accumulated. The key role in healing of wound defects belongs to fibroblasts due to the secretion of the extracellular matrix components, metabolites, signal factors for the surrounding cells, and tissue metabolism regulation. The paper presents the results of the study of the effect of *Bifidobacterium bifidum* supernatant (10 μl/mL) on the morphological and functional properties of human fibroblasts in real time during the *in vitro* experiment. In our work, we used the reference strain *B. bifidum* 791 (All-Russian Collection of Industrial Microorganisms of the State Research Institute for Genetics and Selection of Industrial Microorganisms “Genetika”, Deposit No. AS-1247) used in the production of the probiotic product “Bifidumbacterin” (ZAO “Ecopolis”, the city of Kovrov), and adult human fibroblasts (cell line LECH-4 (81)) (laboratory of cell cultures ENIIVI, the city of Yekaterinburg). Structural and functional studies were conducted on co-culture days 1, 3, 7, 14, 21, and 28. The products of *B. bifidum* secondary metabolism have a stressful effect on the morphological and functional state of fibroblasts on the first day. The processes of proliferation are stimulated in the culture in the experiment (2.67±0.24) compared with the control group (0.75±0.15) ($p < 0.01$) without blocking apoptosis in the cell. This leads to the increase in the production of extracellular matrix proteins, both collagen (pg/mL) (400±19 against 110±25 in the control group), and elastin (ng/mL) 395±30 and 125±29). Co-culture of fibroblasts within 24 hours in the experimental sample leads to a massive “release” of the CD44 receptor ($p < 0.05$), compared to the control group which is confirmed by phenotypic changes (r = 0.66). The decrease of $CD105^+$, $CD44^+$ receptors ($p < 0.05$), compared with the control group and the increase of $CD29^+$ expression ($p < 0.05$) is observed on days 1 and 3. Activated fibroblasts have an altered secretory phenotype that produces cytokines of various types such as TGF-β (r = 0.78), IL-6 (r = 0.57), IL-1β (r = 0.75), IL-8 (r = 0.63). The maximum adaptation of cells in the experimental system is registered on the 7th day, which correlates with morphometric (r = 0.59) and cytometric (r = 0.71) studies. The received data contribute to understanding of the mechanisms of the immunoregulatory influence of normal biota (in the bifidobacteria model) on the repair and regeneration processes.

Keywords: fibroblasts, Bifidobacterium bifidum, cytokines, collagen, elastin, flow cytometry

Introduction

Wound healing is a highly dynamic process, which involves complex interactions of extracellular matrix molecules, soluble mediators, and various resident cells [11]. The key role in wound repair belongs to fibroblasts that provide collagen to the new extracellular matrix and produce a number of cytokines, chemokines and growth factors in response to tissue damage [12]. Currently, there is an active search for exogenous stimulators of repair and regeneration processes. In the recent decades, some data on the immunotropic activity of bifidobacteria have been accumulated [8]. Our pilot study confirms the immunomodulatory function of *Bifidobacterium bifidum*, which regulates the balance between Th1 and Th2 immune response [5]. The published studies have shown that probiotics, especially the *Bifidobacterium bifidum* genus, can stimulate fibroblast migration and proliferation as well as the formation of new blood vessels during the wound healing process [7]. In view of the above, the interest in further research into the reparative and regenerative properties of probiotic bacteria is obvious.

The aim of the research was to study the effect of *Bifidobacterium bifidum* (*B. bifidum*) supernatant on the morphological and functional characteristics of human fibroblasts in real time during the *in vitro* experiment.

Materials and methods

The reference strain *B. bifidum* 791 (All-Russian Collection of Industrial Microorganisms of the State Research Institute for Genetics and Selection of Industrial Microorganisms “Genetika”, Deposit No. AS-1247) utilized in the production of “Bifidumbacterin” probiotic was used in our study. The *B. bifidum* supernatant was obtained by the method described previously [13].

Adult human fibroblasts (LECH-4(81) cell line) (Laboratory of Cell Cultures, Yekaterinburg) were cultured in DMEM/F12 medium containing 2 mM L-glutamine and 10% fetal calf serum. Fibroblasts were seeded on Petri dishes at a density of 1×10^6 cells/cm^2 and placed in standard conditions of CO_2 incubator B 52 (BINDER GmbH, Germany). Live imaging of fibroblasts was performed using a Nikon Ts2 inverted microscope (Nikon Corp, Japan) using NIS-Elemnts D 5.30.00 (Assembly 1531) 64-bit software with a Nikon Ts2 inverted microscope (Nikon Corp, Japan), and NIS-Elements D 5.30.00 (assembly 1531) 64-bit software.

The proliferative activity was studied according to the time of cell population doubling and the state of their cell cycle [6]. The expression of specific surface cell markers was assessed by fibroblasts immunophenotyping using the CytoFLEX flow cytometer. For this purpose, the cells were removed from the surface of the plastic using a 0.25% trypsin-EDTA solution with Hanks salts (PanEco, Russia), washed out of the culture medium and the enzyme, and then their number was counted.

The monoclonal antibodies used in this work were as follows: CD105-FITC (endoglin, a coreceptor to transforming growth factors (TGF) β1 and β3), CD90-FITC (thymocyte differentiation antigen 1), CD45-FITC (common leukocyte antigen), CD34-FITC (intercellular adhesion molecule), HLA-DR FITC (major histocompatibility complex DR molecule), CD14 (membrane glycosyl-phosphatidylinositol-related protein expressed on the surface of monocytes), CD44-FITC (transmembrane glycoprotein), and CD29-FITC (β1 integrin subunit) (BD Bioscience, USA). The cell suspension was incubated with antibodies in the dilutions recommended by the manufacturer for 30 min at room temperature. After that, it was washed from the antibodies not bound to the antigen by the CellWash solution (Becton Dickinson, USA). Cell viability was determined using the colorimetric analysis of MTT 3-(4.5-dimethylthiazol-2-yl)-1.5-diphenyltetrazolium bromide [1].

Fibroblast supernatants were selected on incubation days 1, 3, 7, 14, 21, and 28, passed through a filter with a pore diameter of 0.22 μm (GE Osmonics, USA), and stored at -80 °C. The concentration of elastin, collagen type I (COL1), and soluble CD44var (v6) was determined by enzyme immunoassay (ELISA) using Invitrogen test systems, Thermo Fisher, USA), according to the manufacturer’s method. The determination of cytokines (IL-1β, IL-6, IL-8, IL-10, TGF-β, IL-18 and IL-19) was conducted by ELISA method: IL-1β, IL-6, IL-8, IL-10, TGF-β, IL-18 and IL-19 using a standard set of reagents by “Protein Contur” Ltd (Russia) in accordance with the manufacturer’s instructions. The results were registered in a Multiskan photometer (Labsystems, Finland).

Preparation of fibroblast culture for experimental conditions

The following factors influence the growth rate and properties of fibroblasts in culture: the number of passages, method of cultivation, type of media and sera used. The obtained cellular population is heterogeneous (small spindle-shaped progenitor cells; larger spindle-shaped maturing cells; large mantle-like mature fibrocytes).

The main pool of cells is in G0-phase, 9.5 to 19% of culture is in the process of constant division, the DNA index is 1.96 on the average, which corresponds to the characteristics of differentiated fibroblasts.

An important feature that characterizes the functional state of the cell population is the synthesis of extracellular matrix proteins. Fibroblasts lose this ability during long cultivation, and the risk of accumulation of chromosomal abnormalities in the cells arises [9]. Considering the above, the fibroblasts were taken into the experiment after 5-6 passages [2].

MTT analysis was carried out for the determination of the optimal dose of *B. bifidum* supernatant. The results obtained by co-culturing of fibroblasts with *B. bifidum* supernatant showed that the maximum cell proliferation was at a supernatant concentration of 10 μl/mL ($p = 0.011$). Each experiment was replicated 3 times with duplication of analytical measurements (n-3).

The design of the experiment:

1. Control group;
2. Co-culture of fibroblasts with *B. bifidum* supernatant at a concentration of 10 μl/mL.

Data distribution was evaluated using the Kolmogorov–Smirnov test (one-sample and two-sample tests). The data were expressed as mean value ± standard deviation, and analyzed using a t-test for the paired sample. $p < 0.05$ was considered statistically significant. The SigmaPlot software (version 12.0) was used for representation of data.

Results and discussion

Fibroblasts *in vitro* are characterized by plasticity and a variety of forms (oval, polygonal, fusiform, dendritic) [4]. The morphology of fibroblasts after 24-hour co-culture with *B. bifidum* supernatant was different from cells cultivated under normal conditions. The cells were in both suspended (dead) and adherent states. The number of viable cells in

the experiment was 48% ±9, and 90% ±3 was in the control group, respectively. Microscopy showed small rounded cells: prefibroblasts with a high proliferative potential. The population in the control group was heterogeneous (small spindle-shaped progenitor cells and larger spindle-shaped maturing cells; large mantle-like mature fibrocytes). After 3 days of co-culturing, spindle-shaped cells, with outflowing thin long processes, and spindle-shaped actively dividing progenitor cells were observed. Both large spindle-shaped maturing cells and mature spindle-shaped fibroblasts were observed after 7 days of co-culturing. From the 14th day of cultivation in the experimental and control samples, cells were spindle-shaped with a large ellipsoidal nucleus.

The proliferation index increased after 24-hour co-culture in the experiment (2.67±0.24) compared with the control group 0.75±0.15 ($p < 0.01$), then gradually decreased by the 7th day (1.13±0.09) that corresponded to the control group (1.03+0.1). The decrease of the proliferation index in the compared groups was observed from the 21st day reflecting the morphological and functional state of fibroblasts. The high proliferation index as well as the increased production of extracellular matrix components can presumably be explained by the mechanisms of cell adaptation to co-culture conditions [3]. Apparently, at the initial stage of co-culturing, the culture medium when supernatant is added is “stressful” for the fibroblasts, and the state of the cells themselves during this period is shocking. Then, some time is spent for the preparation and carrying out of adaptive “measures” due to which it is possible not only to avoid complete death of cells, but also to create conditions for stimulation of proliferative process, reducing high, at first obviously “cytotoxic” effect.

The functions of fibroblasts are as follows: the synthesis of extracellular matrix proteins (collagen of several types), the main component of the intercellular substance (elastin), and the metabolism of hyaluronic acid, as evidenced by the concentration of soluble CD44.

The reliable increase in the concentration of both collagen and elastin in the supernatant when compared with the control group is observed during the first day of co-culturing (Table 1). The increase in the synthesis of extracellular matrix materials by the fibroblasts in the control group and their decrease in the experimental group to the level of control indices is observed by the 3rd day. The decrease in the synthesis of collagen and elastin to the minimum indices is registered on the 28th day and observed in both studied groups during subsequent cultivation (7, 14, 21 days). The results obtained show the aging of the fibroblast culture in the studied groups, which is confirmed by morphological changes [10]. Co-culturing fibroblasts in the experimental sample for a day leads to a massive “release” of the CD44 receptor (Table 1), in contrast to the control, which is confirmed by phenotypic changes ($r = 0.66$) (Figure 1). However, on the 3rd day we observed a decrease in the studied marker in the supernatant, which reflects, in our opinion, the adaptation of the fibroblast culture to the new conditions of cultivation.

Maximum cell adaptation in the experimental system was recorded on day 7, which correlates with morphometric ($r = 0.59$) and cytometric ($r = 0.71$) studies. Aging of fibroblast culture was detected starting from the 21st day of cultivation, which was confirmed by a sharp increase in the concentration of soluble receptor CD44 ($r = 0.88$). The stimulation by the supernatant (10 μl/mL) leads to significantly higher levels of cytokine release in the first day of co-culture compared to unstimulated cells. Among the tested 7 pro- and anti-inflammatory cytokines, the results of only IL-1β, IL-6, IL-8, and TGF-β were significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The immunophenotype of fibroblasts allows evaluating their antigenic profile. The subpopulation composition of the fibroblast culture is characterized by the expression of mesenchymal (CD44, CD29, CD90, CD105) and the absence of epithelial, hematopoietic leukocyte and activation markers (CD14, CD34, CD45, and HLA-DR) [9].

All mentioned above has been confirmed by the analysis of the expression of specific cell surface markers during co-culture with *B. bifidum* supernatant. A decrease of $CD105^+$, $CD44^+$ receptors ($p < 0.05$), compared with the control group, and an increase of $CD29^+$ expression ($p < 0.05$) was observed on the

TABLE 1. CHANGES IN THE CONCENTRATION OF TYPE I COLLAGEN, ELASTIN, SOLUBLE CD44 BY FIBROBLAST CULTURE UNDER CONDITIONS OF CO-CULTURE BY *B. bifidum* SUPERNATANT

		Days of cultivation					
		1	3	7	14	21	28
Type I collagen, pg/mL	Experiment	400±19*	362±24#	148±13* #	54±10#	17±4#	5.0±0.2* #
	Control	110±25	364±25#	210±21#	49±11#	18±2#	0#
Elastin, ng/mL	Experiment	395±30*	190±17* #	92±19#	32±5#	10±3#	3.0±0.2* #
	Control	170±19	262±24#	108±13#	34±10#	12±2#	0* #
Soluble CD44, ng/mL	Experiment	19±5*	7.0±1.2* #	4.0±0.02#	9.0±1.2#	18.0±1.9#	25.0±4.2#
	Control	5.0±0.4	3.00±0.03#	3.0±0.06	10.0±1.4#	15.0±2.2#	29.0±5.4#

Note. *, reliable differences between control and experimental groups, $p < 0.05$, $n = 15$. #, reliable differences from days 1 to 28 in experimental and control groups, $p < 0.05$, $n = 15$.

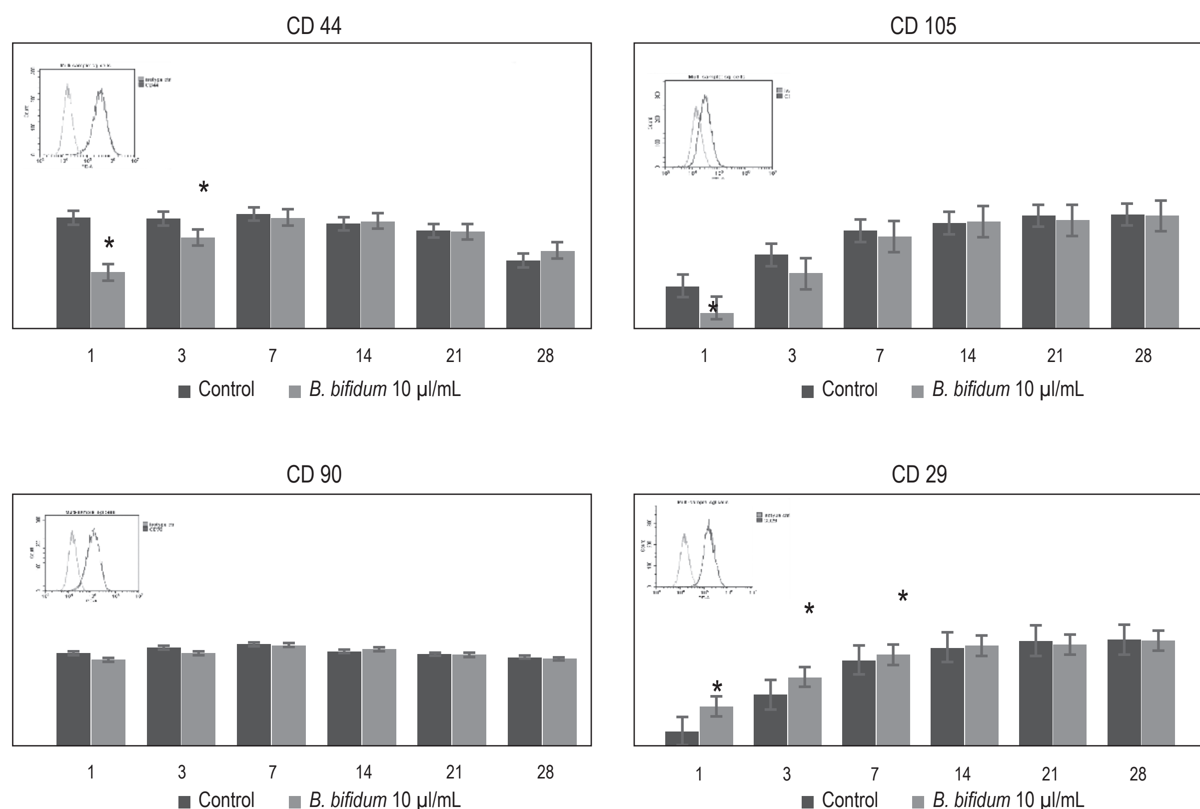

Figure 1. Expression of CD90 CD29 CD44 CD105 markers during co-culture with *B. bifidum* supernatant

Note. The dependence of the expression level of mesenchymal markers over fibroblasts on the time of incubation of fibroblasts with *B. bifidum* (10 µl/mL) supernatant. Isotype control group is represented by cells treated only by secondary fluorescently labeled antibodies only. Control group does not contain *B. bifidum* supernatant. The results of the average value from three independent experiments are presented; error bars correspond to the standard error of the mean. Statistical reliability of differences between the experimental and control groups was tested using Student's t-test, *corresponding to $p < 0.05$.

1st and 3rd days. Recovery of the number of CD105+, CD44+, CD29+ receptors was observed by the 7th day of co-culturing of fibroblasts with the supernatant (Figure 1). Activated fibroblasts have an altered secretory phenotype producing cytokines TGF-β (r = 0.78), IL-6 (r = 0.57), IL-1β (r = 0.75), IL-8 (r = 0.63), which promote proliferation and mediate the recruitment of other cell types to the damaged tissue. Fibroblasts classically function in acute wound healing by becoming "reversibly" activated, secreting extracellular matrix to heal the wound.

Conclusion

The products of the secondary metabolism of *B. bifidum* have a stressful effect on the morphological and functional state of fibroblasts on the first day of co-culturing. They stimulate proliferation processes; do not block apoptosis in the cell, which leads to increased production of extracellular matrix proteins and cytokines of different action, apparently potentiating the processes of tissue repair and regeneration.

Conflicts of interest

The authors have no conflicts of interest to declare.

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