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Study of the anti-inflammatory and immunotropic activity of the secretome from multipotent mesenchymal stromal cells induced by erythropoietin, valproic acid or dexamethasone *in vitro*

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ABSTRACT

The aim of this study was to evaluate the effect of treatment with valproic acid, erythropoietin, and dexamethasone on the anti-inflammatory and immunosuppressive activity of the secretome of adipose-derived multipotent mesenchymal stromal cells (MMSCs) in an *in vitro* experiment.

Materials and methods. MMSCs were isolated from the fat of 6 healthy donors. The cells were grown in the culture up to passage 4. Then they were treated with valproic acid, erythropoietin or dexamethasone for 3 hours, washed from preparations, and incubated in a serum-free medium for 48 hours. Some of the cells were not treated with preparations. Supernatants from the cell cultures were concentrated by ultrafiltration, and protein standardization was performed using a nanophotometer. Then the supernatants were sterilized and added to mononuclear cells from peripheral blood of 8 healthy donors. The mononuclear cells were isolated by Ficoll density gradient centrifugation according to the standard protocol. Concentrations of TNFα, IL-2, IL-4, IL-6, IL-10, and IFNγ cytokines in 24-hour cultures and IL-9, IL-10, IL-17A, and IL-21 cytokines in 48-hour cultures were determined using multiplex analysis.

Results. The production of IL-2, IL-6, TNFα, and IL-10 was reduced by the secretome of MMSCs treated with valproic acid. The production of IL-2, IL-6, and TNFα decreased during incubation of the mononuclear cells with the secretome of MMSCs treated with erythropoietin. The secretome of dexamethasone-treated MMSCs suppressed the production of IFNγ, IL-1β, IL-1ra, IL-2, IL-6, IL-9, IL-10, and IL-17A. No statistically significant differences were revealed in the production of IL-4, IL-5, IL-9, and IL-21.

Conclusion. Among the studied inducers, dexamethasone enhanced the anti-inflammatory and immunosuppressive activity of MMSCs the most, which was manifested through the effect of their supernatants on peripheral blood mononuclear cells.

Keywords: multipotent mesenchymal stromal cells, valproic acid, erythropoietin, dexamethasone, cytokines, inflammation

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Conformity with the principles of ethics. All study participants signed an informed consent. The study was approved by the Ethics Committee at the Belgorod State National Research University (Protocol No. 1 of 24.01.2020).

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Изучение противовоспалительной и иммунотропной активности секретома мультипотентных мезенхимальных стромальных клеток, индуцированных эритропоэтином, вальпроевой кислотой или дексаметазоном *in vitro*

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РЕЗЮМЕ

Цель. Исследовать влияние обработки вальпроевой кислотой, эритропоэтином и дексаметазоном на противовоспалительную и иммуносупрессивную активность секретома мультипотентных мезенхимальных стромальных клеток (ММСК) жировой ткани в эксперименте *in vitro*.

Материалы и методы. ММСК выделяли из жира шести здоровых доноров. Клетки растили в культуре до четвертого пассажа, затем обрабатывали вальпроевой кислотой, эритропоэтином или дексаметазоном в течение 3 ч, отмывали от препаратов и инкубировали в бессывороточной среде в течение 48 ч. Часть клеток не обрабатывали препаратами. Супернатанты от культур клеток сконцентрировали ультрафильтрацией, стандартизировали по содержанию белка с помощью нанофотометра, стерилизовали и добавляли к мононуклеарам из периферической крови восьми здоровых доноров. Мононуклеары выделяли в градиенте плотности фиколла по стандартному протоколу. Концентрации цитокинов фактора некроза опухоли альфа (TNFα), интерлейкина (IL) -2, IL-4, IL-6, IL-10, интерферона гамма (IFNγ) в суточных культурах и IL-9, IL-10, IL-17A, IL-21 в 48-часовых культурах определяли с помощью мультиплексного анализа.

Результаты. Продукция IL-2, IL-6, TNFα, IL-10 снижается под действием секретома от обработанных вальпроевой кислотой ММСК. Продукция IL-2, IL-6, TNFα уменьшается при инкубации мононуклеаров с секретомом клеток, обработанных эритпропоэтином. Секретом обработанных дексаметазоном ММСК подавляет продукцию IFNγ, IL-1β, IL-1ra, IL-2, IL-6, IL-9, IL-10, IL-17A. Статистически значимых различий по изменению продукции IL-4, IL-5, IL-9, IL-21 не выявлено.

Заключение. Среди изученных индукторов дексаметазон показал себя более активным в усилении противовоспалительной и иммуносупрессивной активности ММСК, выраженной через влияние их супернатантов на мононуклеары периферической крови.

Ключевые слова: мультипотентные мезенхимальные стромальные клетки, вальпроевая кислота, эритропоэтин, дексаметазон, цитокины, воспаление

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

The anti-inflammatory activity of multipotent mesenchymal stromal cells (MMSCs) has been confirmed by numerous studies. Currently, much attention is paid to the regulatory effects of MMSCs, which are determined by the action of biologically active substances in their secretome. Thus, the secretome can be an effective alternative to the use of stem cells and is a good basis for development of innovative drugs. However, the possibility of changing the functional activity of MMSCs and, hence, the composition of their secretome after pharmacological stimulation with erythropoietin (EPO), valproic acid (VA), and dexamethasone (DEX), has not yet been realized in the world [1]. There is evidence that EPO increases the survival rate of MMSCs when they are injected together. EPO also reduces the inflammatory microenvironment of diabetic foot ulcers. The mechanism consists in inhibition of the release of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) by the cells [2].

Therefore, EPO is a potential regulator of the functional activity of cells with receptors for it, including stromal cells [2], however, the exact effects remain poorly studied. It has been shown that the use of VA in therapy inhibits proliferation and differentiation of MSCs, as well as release of proinflammatory cytokines [3]. With the administration of lipopolysaccharide (LPS) and VA to dogs, VA has been shown to reduce the production of proinflammatory cytokines. It is known to increase the anti-inflammatory activity of embryonic fibroblasts under the influence of DEX [4].

The aim of the research was to study the possibility of changing the anti-inflammatory and immunosuppressive activity of the secretome of adipose-derived MMSCs after treatment with VA, EPO, and DEX *in vitro*.

MATERIALS AND METHODS

All individuals included in the study signed an informed consent. The study was approved by the Ethics Committee at Belgorod State National Research University (protocol No. 1 of 24.01.2020). Mononuclear cells (MNCs) from peripheral blood of 8 healthy volunteers were isolated by Ficoll density gradient centrifugation. MMSCs were isolated from the adipose tissue of 6 donors using collagenase type II. MMSCs were grown up to passage 4 in the Minimum Essential Medium (MEM)-**α** with 10% fetal bovine serum under standard conditions of a gas incubator (humid atmosphere, 5% CO_2, 37 °C). Then the cells were seeded in culture plates and treated with 1 IU / ml of EPO (Sandoz, Slovenia), 20 **μ**g / ml of VA (Merck, USA) or 10 **μ**mol / ml of DEX (CSPC Ouyi Pharmaceutical, China) for 3 h. The cells were washed from the preparations and incubated in a serum-free medium for 48 h under standard conditions.

Some of the MMSCs were not treated with any of the pharmacological agents. Before adding to the culture plate, supernatants from MMSC cultures were concentrated using Vivaspin 15 R centrifugal concentrators (Sartorius, Germany) with molecular weight cut-off (MWCO) of 3 kDa, standardized for the total protein content (1 mg / ml) using a nanophotometer (Implen, Germany), sterilized by filtration, and then added to the plates with MNCs. To stimulate MNCs, 10 μg / ml of phytohemagglutinin (PanEco, Russia) and 100 ng / ml of LPS (Merk, USA) were added. After 24 h and 48 h, the plate was centrifuged, and the supernatant was taken for the study of cytokine concentrations. Concentrations of cytokines TNF-α, interleukin (IL)-2, IL-4, IL-6, IL-10, and interferon gamma (IFN-**γ**) in daily cultures were determined using Bio-Plex Pro Human Cytokine 8-Plex and Human Ultrasensitive Cytokine Magnetic 10-Plex Panels (Bio-Rad, Invitrogen, USA). Concentrations of IL-9, IL-10, IL-17A, and IL-21 in 48-hour cultures were determined using the Th9/Th17/Th22 Cytokine 7-Plex Human ProcartaPlex Panel (Invitrogen, USA) according to the instructions on the MAGPIX multiplex reader (Luminex, USA). The concentration of cytokines produced by stimulated MNCs was taken as a control. To confirm the MMSC phenotype, the cells were stained with antibodies to CD105, CD90, CD73, CD31, CD45, and CD34 (BD, Beckman Coulter, USA). Mouse IgG1 conjugated with BV 421 (BD, USA) was used as an isotype control. The expression of markers was determined on

the FACSCanto II flow cytometer with BD FACSDiva software (BD, USA).

Statistical processing of the results was carried out using the SPSS Statistics 17.0 software (IBM, USA). The data obtained were checked for normal distribution using the Shapiro – Wilk test. Descriptive statistics were represented by the median and the interquartile range *Me* $[Q_1–Q_3]$. Statistically significant differences were calculated using the Mann – Whitney U test and the Kruskal – Wallis test; the differences were considered statistically significant at $p < 0.05$.

RESULTS

The secretome from DEX-stimulated MMSCs significantly ($p < 0.05$) reduced the production of the regulatory cytokine IFN-γ (Table). Incubation of MNCs with the secretome from MMSCs treated with EPO and VA did not reveal a statistically significant decrease in the level of this cytokine ($p > 0.05$). A decrease in the concentration of IL-2 under the influence of VA, EPO, DEX, and joint incubation with the MMSC secretome was significant in all cases, regardless of the presence or absence of preliminary MMSC treatment (Table). However, the greatest effect in reducing the production of this cytokine was shown by the secretome from DEX-treated MMSCs ($p < 0.05$). The secretome from DEX-treated MMSCs contributed to a decrease in the production of IL-4 and IL-5 by 1.3 and 1.6 times, respectively ($p > 0.05$). A tendency toward suppression of IL-21 production was also noted ($p > 0.05$). The production of proinflammatory cytokines decreased after incubation with the secretome from DEX-treated MMSCs: TNF-α – by 6.0 times, IL-1β – by 2.5 times, IL-6 – by 1.5 times, IL-9 – by 5.6 times, and IL-17A – by 3.2 times (Table, $p < 0.05$).

A pairwise analysis revealed differences between the decrease in TNF-α, IL-1β, and IL-17A after the administration of the secretome from DEX-treated MMSCs and secretomes from native MMSCs and those treated with EPO and VA ($p < 0.05$). A decrease in the IL-6 concentration under the influence of all secretomes was statistically significantly smaller ($p < 0.05$) than in the control. However, the pairwise analysis did not reveal any differences in the co-incubation of secretomes from stimulated and native MMSCs. A decrease in the production of anti-inflammatory cytokines IL-1ra and IL-10 (Table) after treatment of the cells with EPO and DEX was noted. This effect was especially pronounced after exposure of MNCs to the secretome of DEX-treated MMSCs ($p < 0.05$).

DISCUSSION

A statistically significant decrease in IFNγ was observed upon incubation of MNCs together with the secretome from DEX-treated MMSCs ($p < 0.05$), which is consistent with the data on the decrease in IFNγ by glucocorticoids and the effect of MMSCs in treatment of imiquimod-induced psoriasis [5]. IL-2 produced by Th1 lymphocytes decreased upon incubation with the secretome from MMSCs treated with DEX ($p < 0.05$). There are conflicting literature data that glucocorticoids have a positive effect on the production of IL-4, IL-10, and IL-13 by Th2 cells, causing a shift toward humoral immunity without immunosuppression. However, most scientific works have shown that synthesis of IgE *in vivo* is declining [6]. The biological functions of IL-21 include induction of an inflammatory T-cell response and suppression of IgE production. It has been shown that treatment of thrombocytopenia with high doses of DEX leads to a decrease in IL-21 [7]. We revealed only a tendency toward a decrease in IL-21 production under the influence of MNC incubation with the secretome from MMSCs. Confirmation of this phenomenon, along with the revealed trend toward a decrease in IL-4 and IL-5, can be used in correction of IgE-related diseases.

IL-1β was significantly reduced after exposure to the secretome from MMSCs treated with DEX. It is known that there is no significant effect of VA on the production of this cytokine [8], while it moderately increases with the addition of EPO [9]. IL-6 decreased after pharmacological treatment of cells, which is consistent with the article [8].

Recent data confirm that Th1, Th2, and Th17 cells have different sensitivity to glucocorticoids [10, 11]. It has been shown that Th1- and Th17-related cytokines are involved in the development of systemic sclerosis [4], and increased expression of IL-17A is observed during the development of inflammation in psoriasis [5]. The authors demonstrated a significant decrease in the production of IL-1β, IL-6, IL-10, IFNγ, TNF, and IL-17A by *in vitro* exposure of MNCs from patients with systemic sclerosis to DEX [4]. The revealed decrease in IL-17A after exposure to the secretome of DEX-treated MMSCs may be used in treatment of systemic sclerosis and psoriasis.

In addition, a tendency toward a decrease in IL-17A under the influence of the secretome from EPO-treated MMSCs was revealed, which can be potentially used in chronic inflammatory diseases, such as colitis [12]. It was shown that the DEX-induced decrease in

Table

Concentration of cytokines produced by different groups of cells (pg / ml), *Me* [Q_1–Q_3]

Cells	Cytokine concentration, pg / mL										
	IFNγ	IL-2	IL-4	IL-5	IL-21	TNFα	IL-1β	IL-6	IL-9	IL-17A	IL-1ra	IL-10
MNC	3.4 [2.3–4.5]*	159 [150.6–171.6]*	187 [155.7–229.2]*	70.7 [33.2–120.7]	1.8 [1.8–1.8]*	45.0 [25.2–65.8]	17.4 [12.2–23.6]*	18186.7 [18111.7–18286.7]	0.1 [0.1–0.3]*	1.6 [0.4–1.6]*	1314.2 [1309–1319.3]	11.7 [7.4–14.4]
MNC stim.	2067.5 [837.6–2737.7]	1330.4 [1255.4–1430.4]	445.8 [370.8–545.8]	141.3 [66.3–241.3]	53.3 [52.1–80.6]	3961.2 [3886.2–4061.2]	9880.1 [8479.8–13256.2]	22955.0 [22820.7–23041.2]	45.3 [23.5–153.7]	308.3 [219.3–698.4]	3996.5 [2821.7–4730.5]	6017 [4495.2–7995.4]
MMSC stim.	103.7 [76.1–122.4]*	75.8 [29.4–84.4]*	98.8 [52.8–127.9]*	34.8 [14.4–47]	23.6 [18.2–24.3]*	40.8 [24.4–62.3]*	185.1 [64.8–329.3]*	6271.9 [1147–7513.4]*	6.4 [5–6.9]*	32.8 [28.1–39.1]*	13.7 [9.9–44.5]*	22.6 [5.7–73.9]*
MMSC + MNC stim.	1567.9 [865–2120.4]	624.3 [616.8–809.8]*	319.6 [312.1–397.9]	137.9 [129.2–167.4]	37.9 [12.6–75.7]	2254.7 [1638–2272.5]*	7848.6 [6251–12121.9]	14703.6 [14380.4–15615.5]*	33.0 [20–99.7]	335.3 [328.7–702.8]	4191.7 [3170.8–4918.3]	5522.9 [4074.3–8209]
MMSC + EPO + MNC stim.	1421.2 [1379–1634.7]	731.8 [720.6–838.4]*	386.1 [361.8–390.7]	144.7 [143.3–154.7]	36.4 [35.1–49.5]	1985.6 [1895.4–2297.6]*	7015.0 [6092.2–7050.6]	14304.2 [14072.9–15377.4]*	15.3 [8.2–24.3]	243.9 [51.8–1089.8]	3659 [2561.3–4317.6]	4618.7 [4125.6–5603.2]
MMSC + DEX + MNC stim.	654.9 [203.5–2036.6]*	252.5 [248.7–264.5]*	331 [245.7–350.2]	80.5 [74.3–86.6]	37.4 [10.4–134.2]	657.1 [652.8–685.8]*⊗	3970.8 [1034.7–4806.9]*⊗	11886.7 [11792.4–15446.3]*	8.1 [5.5–9.8]*	96.6 [65.5–261.3 7]*⊗	1924.4 [1312.1–2169.3]*	2457.4 [2085.2–2984]*
MMSC + VA + MNC stim.	1371.8 [1157.3–1577.2]	756.8 [680.1–798.4]*	353.8 [352.3–374.7]	158.9 [132.5–174.3]	37 [34.6–74.7]	2389.5 [1897.1–2449.1]*	6281.3 [6178.3–6387.6]	14755.9 [12630.9–14819.1]*	17.0 [11.7–25.7]	400.7 [146.7–552.3]	4303.5 [3012.5–078.1]	4235.7 [3710.2–4764.3]*

Note: * – statistical significance of the difference in comparison with MNC stim. ($p < 0.05$), ⊗ – statistical significance of the difference in comparison with parameters of other secretomes ($p < 0.05$).

TNF-α was less pronounced after high doses of LPS than after 0.1 ng of LPS. On the contrary, the effect of DEX on IL-10 secretion is biphasic: stimulation at lower doses of LPS and inhibition at higher doses of LPS [13]. We used 10 μmol / ml of DEX and 100 ng / ml of LPS, which is 10 times more than in the cited source, but the effects turned out to be natural: a decrease in the production of TNFα and IL-10. This suggests that the secretome from DEX-treated MMSCs has a more pronounced anti-inflammatory effect than that of the native MMSCs.

Glucocorticoids are known to reduce the production of IL-1β. In this study, the greatest decrease in the production of IL-1β and TNFα was observed under the effect of the secretome from DEX-treated MMSCs ($p < 0.05$), which shows the ability of glucocorticoids to exert an indirect anti-inflammatory effect through the influence on stromal cells. It is known that under the influence of DEX, in the blood serum of patients with thrombocytopenia, the production of TNFα significantly decreases [7], and in patients with Crohn's disease, TNFα and IL-6 decrease and genes associated with phagocytosis are inhibited, which can lead to persistent infection [14]. It may be possible to use glucocorticoids to suppress synthesis of proinflammatory cytokines indirectly, using DEX-treated MMSCs and their secretome, avoiding the increased risk of developing infectious diseases. A decrease in production of IL-9, one of the factors in mast cell differentiation, under the effect of the secretome from DEX-treated MMSCs, can be used in allergic and autoimmune inflammation.

Scientists have shown that bone marrow MMSCs reduce the production of IL-9 by MNCs in patients with rheumatoid arthritis *in vitro* [14]. There is evidence that EPO has no effect on IL-10 production [9] and exerts a suppressive effect on TNFα synthesis [6]. This is consistent with our data: a significant decrease in TNFα under the effect of EPO and a tendency toward IL-10 suppression. It has been shown that DEX reduces IL-10 production by LPS-stimulated MNCs [6]. In this study, the level of anti-inflammatory cytokines produced by MNCs under the effect of EPO and the secretome from EPO-treated MMSCs decreased ($p > 0.05$). This effect can be studied in more detail and, if confirmed, used in therapy. As shown in the article [13], IL-1ra production is reduced by DEX, which is consistent with our data. However, this effect is stronger when using the secretome of DEX-treated MMSCs, which suggests possible limitations of its anti-inflammatory effect with long-term use.

CONCLUSION

Significant suppression of IL-2, IL-6, and TNFα production under the effect of secretomes from MMSCs previously stimulated by EPO and VA was shown. However, this effect was more pronounced when cells were treated with DEX. An increase in the anti-inflammatory and immunosuppressive activity of the secretome from MMSCs after preliminary stimulation with DEX at a concentration of 10^{-5} mol / l was found. This effect was observed in the form of a decrease in the production of regulatory cytokines IFNγ and IL-2 and proinflammatory cytokines TNF-α, IL-1β, IL-6, IL-9, and IL-17A by MNCs under the effect of this MMSC secretome. A significant decrease in the IL-10 and IL-1ra production suggests possible limitations in long-term therapy for inflammatory diseases using the studied secretome.

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Golubinskaya P.A. – carrying out of experiments, analysis and interpretation of data, drafting of the article. Puzanov M.V. – critical revision of the manuscript for important intellectual content. Sarycheva M.V. – analysis and interpretation of data. Burda S.Yu. – analysis and interpretation of data, drafting of the article. Nadezhdin S.V. – provision of methodological consultation, critical revision of the manuscript for important intellectual content. Korokin M.V. – research leadership, final approval of the manuscript for publication. Burda Yu.E. – conception and design, control over the quality of research results.

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