DOI 10.1007/s10517-017-3928-2
Bulletin of Experimental Biology and Medicine, Vol. 164, No. 1, November, 2017 IMMUNOLOGY AND MICROBIOLOGY

Effects of Bone Marrow Multipotent Mesenchymal Stromal Cells and Their Secretion Products on the Cellular Composition of the Thymus and Spleen of Female Wistar Rats with Experimental Chronic Inflammation of the Internal Genitals

T. I. Dergacheva[1], A. V. Shurlygina[1,2], E. V. Mel'nikova[2], O. B. Gritsyk[2], M. V. Tenditnik[2], O. V. Poveshchenko[1], and V. I. Konenkov[1]

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 163, No. 7, pp. 89-94, July, 2017
Original article submitted February 22, 2017

The effects of bone marrow multipotent mesenchymal stromal cells and their secretion products on the subpopulation composition of thymic and splenic lymphocytes were studied in female Wistar rats with experimental chronic inflammatory process in the internal genitals. Stromal cells and medium conditioned by these cells in different administration routes (intravenous or lymphotropic injection) produces different modulating effect on blood leukocyte count and on subpopulation composition of the splenic and thymic lymphocytes. The most manifest anti-inflammatory effect was observed after lymphotropic injection of multipotent mesenchymal stromal cells creating a high concentration and long persistence of the factors produced by these cells in the focus of inflammation.

Key Words: *multipotent mesenchymal stromal cells; lymphocytes; inflammation; thymus; spleen*

Inflammatory diseases of the pelvic organs rank first in the structure of obstetrical and gynecological morbidity [1]. Despite the use of various therapies, inflammatory diseases take a chronic course in 70% cases and often lead to infertility, ectopic pregnancy, and chronic pelvic pain [5,6,12]. The inflammatory process is paralleled by quantitative and qualitative changes in the subpopulation composition of lymphocytes in the inflammatory focus [3]. Therapy with multipotent mesenchymal stromal cells (MMSC) is regarded as a prospective trend in therapy for many diseases [2]. The efficiency of cell therapy depends on the functional activity of the transplanted cells and their capacity to migrate to certain tissues and organs [9]. Bone marrow MMSC are characterized by low immunogenic activity, they modulate the inflammatory reactions and immune functions [11,12], due to which they can be used in therapy for chronic inflammatory diseases. We have previously shown that bone marrow MMSC and media conditioned by these cells (CM) administered via different routes cause opposite effects in the subpopulation composition of the thymus, spleen, and lymph node cells [4].

We study the relationship between the route of administration of bone marrow MMSC and bone marrow MMSC CM and the cell composition of immune organs in experimental chronic inflammation of the internal genitals in female Wistar rats.

MATERIALS AND METHODS

Experiments were carried out in accordance with the European Convention for Protection of Vertebrates

[1]Research Institute of Clinical and Experimental Lymphology; [2]Research Institute of Physiology and Basic Medicine, Novosibirsk, Russia. ***Address for correspondence:*** dr-tanja@yandex.ru. T. I. Dergacheva

used for Experimental or Other Research Purposes. The study was carried out on female Wistar rats (260-300 g; *n*=40) fed standard laboratory diet with free access to water. The animals were distributed into 8 groups, 5 per group.

Group 1 were intact animals; group 2 were rats with experimental genital inflammation (day 21 after inflammation induction). Groups 3-8 consisted of rats with chronic inflammation, which received a single injection of MMSC in a dose of 2×10^6 cell/0.1 ml medium intravenously (group 3), lymphotropic MMSC, injected under the vaginal mucosa (group 4), MMSC-CM, 0.1 ml intravenously (group 5) and lymphotropically (group 6), and 0.1 ml saline intravenously (group 7) and lymphotropically (group 8).

Inflammation was induced by injection of a 24-h culture of *S. aureus*, strain 24943, in a dose of 3×10^6 bacterial cells (according to opacity standard [8]) under the vaginal mucosa. MMSC were collected from the femoral bones of 5 rats [7]. Passage 2-4 MMSC were used. Nine days after injection of MMSC and MMSC-CM, the animals were decapitated under light ethaminal narcosis. The blood was collected from the decapitation wound, the spleen and thymus were collected. Cell suspension from the organs was made. The cells were then treated with FITC-labeled monoclonal antibodies to surface lymphocytic antigens CD3, CD4, CD45RA, and CD11b/c and PE-labeled monoclonal antibodies to CD25 and CD8 (BD Pharmingen). In order to evaluate the proportion of cells in various phases of the cell cycle, the cell suspension was processed by PI. The samples were analyzed by flow cytofluorometry (FACSCalibur; Becton Dickinson).

The data were statistically processed using Statistics 6.0 software. The arithmetic mean (*M*), standard error (*SE*), and standard deviation (*SD*) were evaluated by the descriptive statistical methods. The results were compared using Student's *t* test, as the data distribution was normal. The differences were considered significant at $p<0.05$.

RESULTS

The percentage of T cells ($CD3^+$) and T helpers ($CD4^+$), percentage and absolute counts of monocyte/macrophages ($CD11b/c^+$) cells in the spleen increased in chronic inflammation, while the percentage and absolute count of B cells ($CD45RA^+$) and of T-regulatory cells (Treg, $CD4^+25^{+high}$) decreased, as did the percentage of splenocytes in apoptosis (<2n). The total count of cells in the organ did not change. In the thymus, the percentage of $CD3^{high}$ cells increased, while that of $CD4^+25^+$ and percentage and absolute count of Treg decreased. The percentage of cells in active phases of cell cycle decreased (>2n) (Tables 1-3). These shifts indicated the development of the cellular type systemic immune reaction to local inflammation and changes in differentiation of T-lymphocytes in the thymus with an increase in the count of the more mature subpopulation $CD3^{+high}$. Lesser counts and percentage of the natural and inducible Treg could be a factor maintaining chronic inflammation and promoting triggering of autoimmune mechanisms, involving the reproductive organs of females in chronic inflammation [10,14].

Intravenous injection of MMSC led to a decrease in the percentage of $CD4^-25^+$ and $CD4^+25^+$ and percentage and absolute count of $CD4^+25^{+high}$ splenocytes. In the thymus the counts of $CD3^{high}$, $CD3^+$, $CD4^+25^+$, Treg cells decreased (Tables 1, 2). The percentage of apoptotic cells decreased in the thymus and spleen (Table 3). The differential leukocyte count of the blood in this group did not differ from that in the model group.

Lymphotropic injection of MMSC (Table 1) led to an increase in the percentage of T-splenocytes and decreased the percentage of activated $CD4^-25^+$ and $CD4^+25^+$ splenocytes. This route of administration led to a decrease in the total cell count in the spleen and absolute counts of monocyte-macrophages and dendritic cells ($CD11b/c^+$), activated splenocytes ($CD4^-25^+$). In the thymus the counts of $CD4^+25^+$ and Treg cells decreased, but less so than in response to intravenous MMSC. However, the absolute count of Treg remained at the level recorded in inflammation (Table 2). Lymphotropic MMSC led to normalization of the counts of apoptotic cells in the thymus and spleen (Table 3). Only lymphotropic MMSC caused shifts in the blood leukocytic formula, which indicated attenuation of the inflammatory process activity: the percentage of segmented and stab leukocytes in the blood decreased (segmented leukocytes: 13.2±0.4% in inflammation and 10.45±0.50% after MMSC; $p<0.05$; stab leukocytes: 2.00±0.12 and 0.40±0.14%, respectively; $p<0.05$).

Hence, MMSC administered by both methods reduced splenocyte activation and the count of $CD4^+25^+$ thymocytes. In other words, both methods of stromal cell transplantation led to an immunosuppressive effect, described previously [11,13]. However, lymphotropic administration caused also a decrease in the monocyte-macrophage/dendritic cell count in the spleen, causing no changes in the content of inducible Treg, and less intensely reduced the content of natural Treg. This pattern of MMSC immunomodulatory effect seemed to be due to persistence of the cell material in tissues of the foci of inflammation, when the injected cells more actively produced anti-inflammatory cytokines IL-10 and TGFβ [11]. All these facts promoted attenuation of the experimental inflammatory process, which was shown by the relevant changes in the blood leukocytic formula.

TABLE 1. Cell Composition of the Spleen in Female Wistar Rats with Experimental Chronic Inflammation of Internal Genitals in Response to Intravenous and Lymphotropic Injection of MMSC and MMSC-CM (*M*±*SE*)

Parameter	Intact control	Inflammation model	Saline		MMSC		MMSC-CM	
			i/v	l/t	i/v	l/t	i/v	l/t
$CD3^+$, %	44.13±0.84	55.62±1.93*	47.45±3.84	46.45±2.53$^+$	50.52±0.92*	56.60±2.25*^{x}	65.38±0.74*$^{+o}$	62.54±1.91*$^{+x}$
$CD4^+$, %	26.93±0.97	34.50±2.08*	27.15±1.08$^+$	26.63±1.87$^+$	27.24±0.90$^+$	26.86±0.74$^+$	30.72±1.32*	27.50±0.71$^+$
$CD4^-25^+$, %	2.53±0.44	2.96±0.20	2.78±0.39	3.23±0.53	1.82±0.32$^+$	1.52±0.21*$^{+x}$	1.06±0.14*$^{+o}$	1.00±0.10*$^{+x}$
$CD4^+25^+$, %	6.90±0.32	7.18±0.45	6.23±0.51	5.90±0.59	5.94±0.17$^+$	5.96±0.27$^+$	3.84±0.16*$^{+o}$	3.52±0.22*$^{+x}$
$CD4^+25^{+high}$, %	1.77±0.19	1.54±0.10	1.65±0.06	1.48±0.31	1.10±0.07*$^+$	1.40±0.11	1.08±0.09*$^{+o}$	0.72±0.07*$^{+x}$
$CD45RA^+$, %	43.47±1.5	34.38±2.03*	40.63±4.30	36.78±1.77*	35.28±0.68*	32.46±2.03*	25.90±1.34*$^{+o}$	24.78±1.53*$^{+x}$
$CD11b/c^+$, %	21.77±0.80	31.42±1.94*	22.00±0.74$^+$	22.80±1.44$^+$	19.90±1.10$^+$	20.30±0.39$^+$	14.18±0.74*$^{+o}$	15.32±1.60*$^{+x}$
Splenic cells, $\times10^6$	1111.82±102.72	957.99±36.65	1211.09±140.40	2517.86±404.88*$^+$	1173.56±136.88	1759.38±333.22$^+$	1609.10±211.29	1837.89±205.52
$CD3^+$, $\times10^6$	489.31±38.85	535.47±37.68	560.43±32.20	1145.48±137.90*$^+$	593.18±70.67	979.06±157.95*$^+$	1052.11±139.19*$^{+o}$	1146.71±122.23*$^+$
$CD4^+$, $\times10^6$	298.61±26.07	330.84±23.82	326.41±31.60	681.20±146.86*$^+$	322.36±44.59	463.31±72.95	486.61±49.04*$^{+o}$	506.46±62.55*$^+$
$CD4^-25^+$, $\times10^6$	27.30±2.07	28.19±1.76	34.19±6.93	76.08±7.17*$^+$	21.22±4.89	25.73±4.79^x	16.93±2.99*$^+$	18.00±2.23*$^{+x}$
$CD4^+25^+$, $\times10^6$	76.71±8.18	68.73±4.73	76.86±13.31	147.65±24.92*$^+$	69.25±7.27	102.45±15.99$^+$	61.66±7.89	64.85±9.41^x
$CD4^+25^{+high}$, $\times10^6$	19.45±2.10	14.62±1.09*	20.14±2.94	37.67±9.85$^+$	12.58±0.82*^{o}	24.20±4.03$^+$	17.19±2.35	13.20±2.13*^{x}
$CD45RA^+$, $\times10^6$	485.49±56.64	326.69±8.96*	505.37±97.9	945.99±197.22*$^+$	415.73±53.37	589.03±146.63$^+$	416.96±56.31	449.07±47.21^{+x}
$CD11b/c^+$, $\times10^6$	240.92±18.77	299.39±16.64	267.89±35.40	563.10±68.47*$^+$	248.53±35.57	354.87±65.15^x	230.39±39.32	279.53±40.08^x

Note. Here and in Tables 2, 3: i/v: intravenous injection; l/t: lymphotropic injection. $p<0.05$ in comparison with *intact control, $^+$inflammation model, ^{o}intravenous saline group, ^{x}lymphotropic saline group.

TABLE 2. Cell Composition of the Thymus in Female Wistar Rats with Experimental Chronic Inflammation of Internal Genitals in Response to Intravenous and Lymphotropic Injection of MMSC and MMSC-CM (*M±SE*)

Parameter	Intact control	Inflammation model	Saline		MMSC		MMSC-CM	
			i/v	l/t	i/v	l/t	i/v	l/t
$CD3^+$, %	90.03±0.12	90.18±1.69	90.35±0.65	89.08±0.21	90.18±2.29	89.30±0.96	79.48±0.56*+o	81.26±1.39*+x
$CD3^{+high}$, %	19.70±0.75	25.24±0.66*	26.90±6.55	25.08±5.20	18.40±1.35+	28.82±4.69	28.18±1.59*	28.46±2.17*
$CD4^+25^+$, %	27.13±0.42	23.22±1.62*	17.87±1.01*+	18.05±2.92*	12.22±1.06*+o	11.10±0.94*+x	13.64±1.90*+	16.16±1.12*+
$CD4^+25^{+high}$, %	3.13±0.17	1.08±0.12*	1.85±0.32*+	2.08±0.45*+	0.66±0.09*+o	0.84±0.10*x	0.68±0.05*+	0.54±0.06*+x
Total cell count, $\times10^6$	1552.48±199.64	1715.56±320.35	2391.37±402.22	1731.93±234.18	1151.99±123.88	2099.77±582.04	1508.47±151.22o	840.63±177.91*+x
$CD3^+$, $\times10^6$	1397.27±177.73	1533.97±266.13	2168.25±378.54	1543.72±210.28	1032.63±98.63o	1872.49±516.19	1094.76±78.66o	686.42±148.12*+x
$CD3^{+high}$, $\times10^6$	306.11±43.09	425.27±68.37	690.44±266.45	443.53±118.2	215.28±32.28+	538.97±111.54	386.69±28.70	246.55±56.9+
$CD4^+25^+$, $\times10^6$	422.56±59.34	402.7±79.4	418.12±55.50	321.92±80.3	140.58±20.52*o	231.20±63.87*	197.43±18.15*+o	135.42±27.98*+x
$CD4^+25^{+high}$, $\times10^6$	49.14±8.27	18.23±3.28*	46.28±14.60	37.89±10.86	7.44±1.05*+o	17.72±5.17*	10.19±1.08*+o	4.23±0.64*+x

TABLE 3. Counts of Cells in Various Cell Cycle Phases in Lymphoid Organs of Rats after Intravenous and Lymphotropic MMSC and MMSC-CM (%; *M±SE*)

Parameter	Intact control	Inflammation model	Saline		MMSC		MMSC-CM	
			i/v	l/t	i/v	l/t	i/v	l/t
Thymus <2n	0.10±0.00	0.08±0.04	0.28±0.09*+	0.40±0.27	0.16±0.02*+	0.08±0.02	0.10±0.03o	0.12±0.04
Thymus 2n	92.07±0.41	94.82±0.39*	91.68±1.04+	90.98±1.44+	92.34±0.77+	92.36±1.02+	91.08±0.33+	93.04±0.36+
Thymus >2n	7.83±0.41	5.08±0.38*	8.05±1.13+	8.63±1.70+	7.50±0.79+	7.56±1.01+	8.82±0.32+	6.84±0.36+
Spleen <2n	0.03±0.03	0.22±0.06*	0.40±0.15*	0.13±0.03*	0.10±0.00*+o	0.08±0.02+	0.06±0.02+o	0.18±0.07
Spleen >2n	3.97±0.35	3.16±0.70	2.58±0.50	3.70±0.85	4.14±0.65	3.50±0.59	2.56±0.43*	5.00±0.80

Intravenous MMSC-CM led to an increase in the splenic content of $CD3^+$ (T cells), decrease in the percentage of activated splenocytes ($CD4^-25^+$), activated T helpers ($CD4^+25^+$), and Treg ($CD4^+25^{+high}$), monocyte-macrophages, and dendritic cells ($CD11b/c^+$), increase in the absolute count of T cells, T helpers (Table 1), and decrease in the percentage of splenocytes in active phases of cell cycle (>2n) and in apoptosis phase (<2n) (Table 3). In the thymus the percentage of $CD3^{+high}$ increased, while the percentage and absolute counts of $CD3^+$ and Treg and absolute count of $CD4^+25^+$ cells decreased (Table 2).

Lymphotropic injection of MMSC-CM led to an increase in the spleen of the percentage of T cells, reduced the percentage and absolute count of activated splenocytes ($CD4^-CD25^+$), activated T helpers ($CD4^+25^+$) and Treg ($CD4^+25^{+high}$), monocyte-macrophages and dendritic cells ($CD11b/c^+$), and B lymphocytes ($CD4RA^+$), and normalized apoptosis (Table 1). In the thymus the percentage and absolute counts of $CD3^+$ and Treg and the absolute count of $CD4^+25^+$ cells decreased; the percentage of $CD3^{+high}$ increased, while the absolute count of these cells (better differentiated T cells) decreased (Table 2).

Intravenous and lymphotropic injections of MMSC-CM caused no shifts in the leukocytic formula of the blood.

Hence, the effects of intravenous and lymphotropic MMSC-CM on the cellular composition of immune organs were similar to MMSC effects. Importantly that both methods led to a decrease in the percentage of inducible Treg in the spleen and in the percentage and absolute count of natural Treg in the thymus. These shifts were not paralleled by changes in the blood leukocytic formula, which indicated a weaker anti-inflammatory effect of MMSC-MC. Presumably, this was explained by a more rapid elimination of the immunomodulating factors — components of MMSC-CM.

The results indicated that one of the probable mechanisms of the biomedical cell product activity in the studied experimental model was modulation of the Treg cells involved in the development of acute and chronic inflammation. The most manifest corrective effect in experimental chronic genital inflammation in female rats was observed in response to lymphotropic MMSC, creating high concentration and long persistence of anti-inflammatory factors, produced by these cells, in tissue of inflammatory focus.

REFERENCES

1. Ankirskaya AS. Bacterial vaginosis: clinical lecture. Akush. Gin. 2008;(Suppl.):13-16. Russian.
2. Biology of Stem Cells and Cell Technologies. Vol. 1. Pal'tsev MA, ed. Moscow, 2009. Russian.
3. Dergacheva TI, Shurlygina AV, Konenkov VI. Impact of different routes of administration of antibiotics on the genital mucosal lymphoid cells in the treatment of acute salpingitis and oophoritis in reproductive-aged women. Ross. Vestn. Akushera-Ginekologa. 2013;13(1):7-11. Russian.
4. Lykov AP, Bondarenko NA, Surovtseva MA, Kim II, Poveshchenko OV, Ishchenko IY, Kabakov AV, Kazakov OV, Poveshchenko AF, Zav'yalov EL, Michurina SV, Konenkov VI. Effect of Natural and 24-h Illumination on Mesenchymal Stem Cells. Bull. Exp. Biol. Med. 2016;162(1):134-137.
5. Krasnopol'skii VI, Buyanova SN, Shchukona NA. Purulent Gynecology. Moscow, 2001. Russian.
6. Kuakov VI. Modern principles of antibacterial therapy in obstetrics, gynecology, and neonatology. Akush. Gin. 2002;(4):3-6. Russian.
7. Lykov AP, Kabakov AV, Poveshchenko OV, Bondarenko NA, Poveshchenko AF, Kazakov OV, Nikonoriva YV, Konenkov VI. Efficiency of therapy by the cellular product of the sharp myocardial infarction at rats of the wistar line according to bioelectric activity of the myocardium. Mezhd. Zh. Prikladn. Fundamental. Issled. 2014;(8-4):78-84. Russian.
8. Starkova EV, Dergacheva TI, Astashov VV. Patent RU No. 2142163. Method for modelling inflammatory diseases in female genital organs. Bull. No. 33. Published November 27, 1999.
9. Poveschenko AF, Shundrin LA, Avrorov PA, Solovieva AO, Miller TV, Zubareva KE, Voloshina TV, Poveschenko OV, Konenkov VI. Molecular technology research of bone marrow cells migration. Sovremen. Naukoemk. Tekhnol. 2015;(11):22-30. Russian.
10. Serov VN, Tsaregrordtseva MV, Kozhin AA. Clinical and immunological factors in the formation of autoimmune inflammatory ovarian insufficiency. Akush. Gin. 2007;(6):28-33. Russian.
11. Freidlin IS. Regulatory t-cells: origin and function. Med. Immunol. 2005;7(4):347-354. Russian.
12. Gardó S. Inflammation of the pelvis minor. Orv. Hetil. 1998;139(36):2115-2120.
13. Wang M, Yang Y, Yang D, Luo F, Liang W, Guo S, Xu J. The immunomodulatory activity of human umbilical cord blood-derived mesenchymal stem cells in vitro. Immunology. 2009;126(2):220-232.
14. Yang WY, Shao Y, Lopez-Pastrana J, Mai J, Wang H, Yang XF. Pathological conditions re-shape physiological Tregs into pathological Tregs. Burns Trauma. 2015;3(1):pii: 1.