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ВЛИЯНИЕ НА ИММУНОКОМПЕТЕНТНЫЕ КЛЕТКИ ПРИ ТЕРАПИИ МЕЗЕНХИМАЛЬНЫМИ СТВОЛОВЫМИ КЛЕТКАМИ В СРАВНЕНИИ С ТЕРАПИЕЙ НА ОСНОВЕ МИКРОВЕЗИКУЛ У МЫШЕЙ С ХРОНИЧЕСКИМ ЗАБОЛЕВАНИЕМ ПОЧЕК

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Резюме. Мезенхимальные стволовые клетки, которые обладают как плюрипотентными, так и иммунорегуляторными функциями, активно изучаются как один из способов лечения во многих областях медицины, включая реабилитацию в нефрологии. Однако в некоторых статьях, посвященных лечению различных органических патологий с помощью МСК, было доказано, что эффекты от применения МСК в регенеративной медицине заключаются в том, что после трансплантации в поврежденные органы они могут ингибировать апоптоз клеток, способствуют ангиогенезу для лучшего кровоснабжения и в некоторых случаях стимулируют местную пролиферацию клеток в поврежденных тканях. Другими словами, про-регенерационные функции были обусловлены их паракринными эффектами без влияния дифференциации.

При введении мышам МСК было показано иммуноподавляющее действие, что в конечном счете приводило к уменьшению воспаления и улучшенной регенерации почек. Одним из механизмов восстановления могут быть МВ – внеклеточные тельца, продуцируемые МСК. Как стало известно из последних сообщений, МВ сохраняют некоторые функции клеток, такие как перенос микроРНК и т.д. Считается, что терапевтический эффект МСК может быть во многом связан с их иммуносупрессорными свойствами.

Целью данного исследования было изучение иммунорегуляторных свойств микровезикул (МСК-МВ, МВ) в сравнении с мезенхимальными стволовыми клетками (МСК). Эксперимент проводился на мышах линии СВА возрастом 3-4 месяца, использовалась модель глицерол-индуцированной хронической почечной недостаточности (ХПН). МСК были получены из костного мозга сингенных мышей большеберцовой и бедренной костей. Клетки культивировались в культуральном матрасе до достижения монослоя. МВ были получены из МСК путем апоптоза при помощи культивирования в бессывороточной среде в условиях депривации кислорода в течении суток. МСК и МСК-МВ вводились в хвостовую вену мышей, забор органов производился на 11-е сутки после введения МСК и МСК-МВ.

Методом проточной цитофлуориметрии оценили состояние клеточного иммунитета у мышей с хронической почечной недостаточностью (ХПН) после введения мезенхимальных стволовых клеток

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(МСК) и полученных из них микровезикул (МСК-МВ) на 11-е сутки эксперимента. В результате наблюдалось снижение количества Т-регуляторных клеток $CD4^+CD25^+Foxp3^+$, повышение содержания $CD4^+CD44^+CD62^+$ и $CD8^+CD44^+CD62^+$ Т-клеток памяти.

Влияние МВ не существенно отличалось от МСК. Это может свидетельствовать о том, что терапевтические свойства МСК обусловлены в большей степени, если не полностью МСК-МВ.

Ключевые слова: мезенхимальные стволовые клетки, внеклеточные пузырьки, микровезикулы, хроническая почечная недостаточность, почечное повреждение, бесклеточная терапия

MESENCHYMAL STEM CELLS COMPARED WITH MICROVESICLES-BASED THERAPY IMPACTS IMMUNOCOMPETENT CELLS IN MICE WITH CHRONIC RENAL DISEASE

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Abstract. Mesenchymal stem cells which have both pluripotent and immunoregulatory functions are being actively studied as one of the treatment ways in many fields of medicine, including rehabilitation in nephrology. However, in some articles were dedicated to the treatment of different organic pathology with MSC's was proved that the only reported effects from the application of MSC's in regenerative medicine are that, after transplantation to damaged organs, in a paracrine manner they may inhibit apoptosis of cells, promote angiogenesis for a better blood supply, and in some cases stimulate cells that have survived in damaged tissues to proliferate in order to replenish dying tissue fragments. In other words, the pro-reparational functions were due to their paracrine effects without the impact of differentiation. Microvesicles are one of the components of this pro-regenerative effect. And although immunoregulatory action has been shown for MSCs, it remains poorly understood for microvesicles.

The aim of this study was to study the immunoregulatory properties of microvesicles (MSC-MB, MB) in comparison with mesenchymal stem cells (MSC). The experiment was carried out on CBA mice 3-4 months old, a model of glycerol-induced chronic renal failure (CRF) was used. MSCs were obtained from the bone marrow of syngeneic mice of the tibia and femur. MVs were obtained by apoptosis by culturing in a serum-free medium under oxygen deprivation conditions. MSC and MSC-MB were injected into the tail vein of mice; organs were harvested on the 11^{th} day after administration of MSC and MSC-MB.

The state of cellular immunity in mice with chronic renal failure (CRF) after the introduction of mesenchymal stem cells (MSC) and microvesicles derived from them (MSC-MB) on the 11^{th} day of the experiment was assessed by flow cytometry. As a result, there was a decrease in the number of T-regulatory cells $CD4^+$ $CD25^+$ $Foxp3^+$, an increase in the content of $CD4^+$ $CD44^+$ $CD62^+$ and $CD8^+$ $CD44^+$ $CD62^+$ memory T-cells.

The effect of MVs was not significantly different from that of MSC. This may indicate that the therapeutic properties of MSCs are determined to a greater extent, if not completely, by MSC-MB.

Keywords: mesenchymal stem cells, microvesicles, microvesicles, chronic renal failure, chronic kidney disease, acellular therapy

Introduction

Mesenchymal stem cells exerting both pluripotent and immunoregulatory functions are actively studied as one of the treatment approaches in many fields of medicine, including rehabilitation in nephrology. However, some articles [3, 10] dedicated to the treatment of different organic pathology with MSCs confirmed that the only reported effects from MSC application in regenerative medicine revealed that, after transplantation to damaged organs, they may in a paracrine manner inhibit apoptosis of cells, promote angiogenesis for a better blood supply, and in some cases stimulate cells survived in damaged tissues to proliferate in order to replenish dying tissue fragments. In other words, the pro-reparational functions were due to their paracrine effects without the impact on differentiation.

In experimental models *in vivo* the immunosuppressive functions of MSCs were proved, which resulted in decreased inflammation and improved kidney regeneration [3].

Extracellular vesicles are subdivided into exosomes, microvesicles, and apoptotic bodies. Exosomes are the smallest vesicles (30-100 nm) released via fusion of multivesicular bodies containing intraluminal vesicles with the plasma membrane. Microvesicles are vesicular structures (0.1-1.0 μm) shed by outward blebbing of the plasma membrane. The largest EVs (1-5 μm) are apoptotic bodies that are formed during the late stages of apoptosis [2] ExMVs are small circular membrane fragments secreted from the endosomal compartment or shed from the cell surface by blebbing of cell surface membrane. Large ExMVs are released during cell-surface budding, and their sizes range from 100 to 1000 nm in diameter, their functions are presented with removing cell unwanted substances, protecting specific types of cells from complement-mediated cytolysis and transferring multiple receptors, proteins, genetic material (including mRNA and microRNA (miRNA)) and lipids [2, 5, 6, 14]. According to the recent publications, ExMVs play a crucial role in cell–cell communication, tissue homeostasis, cell differentiation, and organ development and remodeling [5]. Microvesicle shedding is also increased after cell activation due to cell injury, proinflammatory stimuli, hypoxia, oxidative stress, or shear stress [1, 6].

Some studies showed that EVs from healthy individuals contain active caspase-3 that was not found in the parental cells, suggesting that caspase-3 might have been removed from the cells to ensure survival. Intriguingly, dying cells release microvesicles bearing the adaptor protein Crkl during the early stages of apoptosis induced by the caspase 3 cascade. These microvesicles were isolated from glomeruli after injury and were shown to induce compensatory proliferation signaling in recipient cells. Taken together, release of microvesicles may remove cell toxic substances but also induce repair in neighbor cells [8].

It was proved, that those MSCs from mouse models have an immunosuppressive effect, which ultimately led to decreased inflammation and improved renal regeneration. One of the recovery mechanisms can be executed by MVs – extracellular bodies produced by MSCs. It is believed that the therapeutic effect of MSCs may be largely mediated by their immunosuppressive properties. Thus, the aim of this study is to assess the contribution of MVs immunoregulatory activity.

Several studies have shown a significant contribution of the immune system to the processes of damage and restoration of renal tissue [15]. In acute kidney injury, regardless of origin, local increased activity of many components of the immune system is observed [4, 8, 9, 15, 4]. Experiments with adoptive transfer of activated macrophages, T- and B-lymphocytes have shown their negative role in pathogenesis of renal failure. At the same time, MSCs have immunoregulatory properties [10], which can also be possessed by the MSCs-produced MVs, but it remains unstudied.

Thus, **the aim of this work** is to study the immunoregulatory properties of MVs produced by MSCs and their contribution to the pro-regenerative effect of MVs on renal tissue.

Materials and methods

Chronic renal failure model

The experiment was carried out on 3-4 month old CBF mice. Chronic renal failure was induced by applying three doses of 8.6 mg /kg of 50% glycerol intramuscularly every 7 days. The resulting rhabdomyolysis has a complex (ischemic, toxic, retention) effect on the kidneys, with a predominant lesion of the proximal tubule epithelium. In this model, necrosis of epithelial cells of the proximal renal tubules is observed, accompanied by a rather significant increase in the level of serum urea. All experiments on animals were carried out in accordance with the "Rules for carrying out work with the use of experimental animals" (Appendix to the order of the Ministry of Health of the USSR No. 755 of 1977).

Isolation of bone marrow mesenchymal stromal cells

Mesenchymal stromal cells were obtained from the bone marrow of syngeneic mice by adhesion to culture plastic template. Bone marrow derived from femur and tibia was suspended in RPMI 1640 culture medium. The bone marrow stroma was mechanically disrupted with a glass homogenizer, cells were washed out twice, and placed in Tissue Cultural Flask 75 sm^2, TPP, in complete growth medium based on RPMI 1640 supplemented with 10% FBS (Fetal Bovine Serum) Qualified, LOT 42Q3684K, Gibco. The non-adherent fraction of bone marrow cells was removed by replacing culture medium, starting from day 3. MSCs showed a typical fibroblast-like phenotype and formed a continuous monolayer by 4 weeks of culture. MSCs were removed with a 0.25% Versen-trypsin solution, washed out twice from the culture medium, and resuspended in physiological saline.

Isolation of microvesicular particles

After reaching a monolayer, some MSCs were subjected to apoptosis by cultivation under oxygen deprivation and serum-free medium for 1 day. Under these conditions, the cells entered apoptosis, their production of microvesicular particles, especially the 100-1000 nm fraction, was markedly elevated. After 24 hours, the supernatant was centrifuged at 2000 g for 15 minutes to remove cell debris followed by cen-

trifugation at 16000 g for 60 minutes at 4 °C. The resulting pellets were resuspended in 100 μl saline. To standardize the MV suspension, protein content was determined by the Bradford method.

Material intake

2 weeks after the last injection, MSC and MSC-MV were inoculated into the tail vein, and on the day 4 and 11 afterwards, splenocytes were collected by using flow cytometry. The cells were frozen according to the protocol. The studies were carried out with the approval of the Ethics Committee of the Republican Scientific and Practical Center for Mental Health (meeting of 04/11/2011).

Thus, 4 groups were randomized: intact mice, CRF, CRF + MSC, CRF + MV, with 10 mice per each group. Negative control – intact mice injected with 0.9% NaCl, positive control – chronic renal failure. MSCs were injected at a dose of 1 million/mouse, by applying similar amount of MVs.

Histological examination

Mouse kidneys were excised and fixed in 4% formalin solution, and dehydrated according to a standard technique and paraffin-embedded. Paraffin sections 4-5 μm thick were obtained by using an HM 340E rotary microtome (Carl Zeiss, Germany), stained with hematoxylin and eosin, Sirius red, and by Mallory's method. Light-optical examination

TABLE 1. CONCENTRATION OF ALBUMIN (ng/ml), CREATININE (ng/ml), FABP1 (ng/ml)

a) Albumin ng/ml				
	Intact	**CRF**	**CRF + MSC**	**CRF + MSC-MV**
4th day	66.11±13.64	492.50±68.32*	153.1±15.5*	179.30±31.10*
11th day		187.00±37.97	187.00±37.97	97.220±1.912*
b) Creatinine ng/ml				
	Intact	**CRF**	**CRF + MSC**	**CRF + MSC-MV**
4th day	2.244±0.173	4.156±0.524*	1.986±0.192*	1.648±0.080**
11th day		4.818±1.749	2.064±0.197	1.5360±0.03523
c) FABP1 ng/ml				
	Intact	**CRF**	**CRF + MSC**	**CRF + MSC-MV**
4th day	0.802±0.216	0.849±0.894	0.844±0.185	0.987±0.188
11th day		1.085±0.367	0.639±0.163	0.780±0.087*

Note. *, p < 0.05; **, p < 0.01.

TABLE 2. CONTENT OF $CD4^+CD25^+FoxP3^+$ REGULATORY CELLS, $CD4^+CD44^+CD62^+$ AND $CD8^+CD44^+CD62^+$ T-CELLS OF MEMORY. DAY 11

a) $CD4^+CD25^+FoxP3^+$			
Intact	**CRF**	**CRF + MSC**	**CRF + MSC-MV**
0.068±0.034	9.788±3.494*	0.293±0.067*	0.5530±0.1714*
b) $CD4^+CD44^+CD62^+$			
Intact	**CRF**	**CRF + MSC**	**CRF + MSC-MV**
6.940±4.326	1.4325±0.6311	27.230±5.321*	3.7325±0.9294
c) $CD8^+CD44^+CD62^+$			
Intact	**CRF**	**CRF + MSC**	**CRF + MSC-MV**
4.370±2.896	1.1750±0.6969	17.0930±0.6043*	3.270±1.529

Note. *, p < 0.05.

and microphotography were performed by using an Axioskop 40 microscope (Carl Zeiss, Germany).

Morphometric analysis of kidney structure was performed on paraffin sections. The following morphometric parameters were determined: the diameters of the superficial renal glomeruli (renal corpuscles); diameter of the collecting ducts and the height of relevant cells, measured in the middle third portion of the renal medulla. Morphometric calculation was performed in the field of view with a 10/25 eyepiece and a 63 objective (Figure 1).

Confocal microscopy

Some cells were stained with Annexin 5 to confirm the onset of apoptosis 24 hours after keeping cells under conditions of oxygen deprivation in serum-free medium (Figure 2). Staining was performed by using TACS® Annexin V-FITC Apoptosis Detection Kit Cat Number 4830-01-K in *in situ* detection protocol for adherent cells.

To do this in per ~ 5 cm^2 sample we used: 10X Binding Buffer 10 μL; Propidium Iodide 10 μL; TACS Annexin V-FITC* 1.0 μL; Distilled water 79 μL, in total volume 100 μL. To prepare 400 μL of 1X Binding Buffer / sample for washing cells after incubation we diluted 10X Binding Buffer in distilled water. Next, we discarded PBS wash from the culture, washed cells twice for 2 minutes in excess volume of 1X Binding Buffer at room temperature. Cells were analyzed immediately under fluorescence microscope using a fluorescent mounting medium.

Statistical processing of results

The data obtained during the study were processed by the one-way ANOVA method, t-test. The values were calculated by using the GraphPad Prism 8 software.

Flow cytometry

During the study BD FACSCanto II and CytoFLEX, Beckman Coulter Flow cytometers were used. Frozen mouse splenocytes were used as well. Standard staining protocol with monoclonal antibody cocktail anti-mouse CD4-FITC, CD8-FITC, CD44-APC, CD62L-PE, CD4-APC, CD25-FITC, FoxP3-PE (all BD Biosciences) was used for detecting extracellular and intracellular markers for further detection of memory T-cells $CD4^+CD44^+CD62L^+$, $CD8^+CD44^+CD62L^+$ and regulatory T-cells $CD4^+CD25^+FoxP3^+$.

Biochemical analysis of the samples

Changes in blood and urinal parameters (creatinine, albumin and FABP1) were examined. The following kits were used:

– BioVision, Creatinine (Mouse) ELISA Kit rev 03/18, Catalog Number E4369-100;

– Abcam, Mouse Albumin ELISA Kit, Catalog Number ab108792;

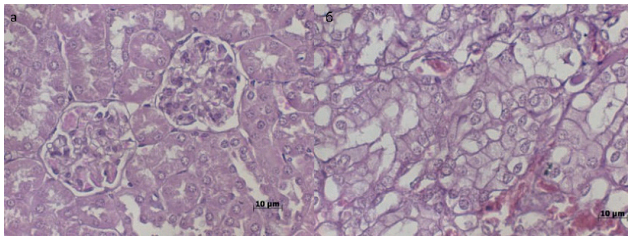

Figure 1. CRF (A) Kidney glomeruli, proximal and distal tubules. (B) The collecting ducts and loops of Henle in the middle part of the medulla of the kidney

Note. Staining with hematoxylin and eosin.

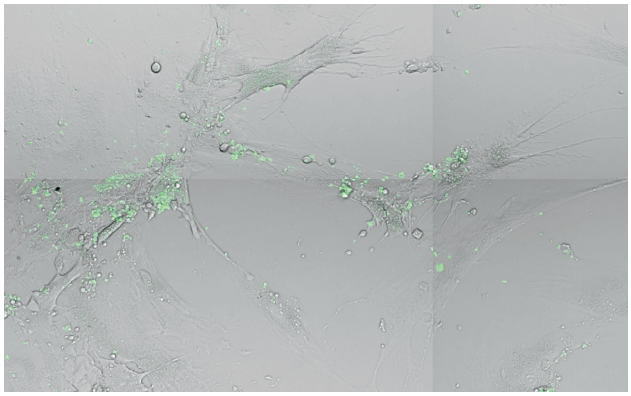

Figure 2. Image shows a typical fibroblast-like structure of MSCs, Annexin V glows green

– RnDSystems, Mouse/Rat FABP1/L-FABP Immunoassay Quantikine® ELISA, Catalog Number RFBP10.

Results and discussion

Mice with induced chronic renal failure (CRF) showed morphological changes in the renal structure and functional biochemical parameters of the excretory system, which were studied after exposure to MSC or MSC-MB on day 4 and 11 after intravenous inoculation of MSCs or MSC-MB. Concentration of urine albumin tended to decrease in mice after exposure to MSC and MSC-MB, but creatine level did not significantly changed (Table 1).

As a marker of kidney damage, the serum creatinine is usually used for calculating the glomerular filtration rate [4]. However, the concentration of creatinine can depend on various factors such as the patient age, medication administration, etc. The creatinine level may not increase immediately, but 72 hours after kidney damage coupled to more than 50% parenchyma dysfunction and significantly changed filtration rate [12]. Proteinuria and albuminuria also not always reflect extent of kidney damage. These parameters point at changes in the glomerular

apparatus, but provide no information about damage to the interstitial tissue and kidney tubules [9].

Biological markers of kidney damage include fatty acids binding proteins (FABPs) also produced in the renal tissue; two types of marker have been revealed: FABP1 (or L-FABP) is synthesized in the epithelial cells of the proximal renal tubules, and H-FABP in the distal renal tubules [12]. L-FABP is not virtually detected in urine of healthy people, but its excretion is increased significantly when interstitial tissue is damaged. Increased expression of L-FABP in tubular cells and its excretion in the urine have been described in animals with ARI (acute renal injury). In 2011, the role of L-FABP was established as an early predictor of kidney damage – in response to massive proteinuria, hyperglycemia, increased blood pressure, upon damage in the proximal kidney tubules coupled to elevated expression of the renal L-FABP gene, which increases excretion of L-FABP in urine. Protein level increased with established ARI of various etiologies, including acute tubular necrosis, sepsis, and nephrotoxin administration. Also, this protein was approved as a new early predictor of kidney damage, published by the Ministry of Health, Labor and Welfare in Japan [12] as well as in case of proximal tubule protein overload.

Kidney morphological examination clearly shows changed characteristic of chronic renal failure, both in the cortex and in the medulla. Dystrophy of cells of the distal nephron of the connecting tubule, hypertrophy of cells of collecting ducts, hypertrophy of cells of Henle's loops are noted, rheological properties of blood are impaired including leakage of blood plasma proteins as well as recorded erythrocyte sludge. On day 4, mice inoculated with MSCs had diameter of the collecting ducts significantly reduced compared to the "CRF" and "CRF + MSC-MV" groups. The height of cells of the collecting ducts was significantly lower compared to "CPI" group. Dystrophy of the cells of the collecting ducts and loop of Henle in the renal cortex and medulla was recorded visually. In addition, blood rheological properties were impaired. The therapeutic effect of MSC microvesicle *vs* MSCs was markedly lower on morphological characteristics: the diameter of the collecting tubules was significantly larger, the height of the collecting tubule cells was bigger. Dystrophy of the cells of the collecting ducts in the middle and lower parts of the medulla, as well as the cells of Bellini's ducts, were also noted visually.

$CD4^+CD44^+CD62L^+$ and $CD8^+CD44^+CD62L^+$ T-memory cells and $CD4^+CD25^+FoxP3^+$ T-regulatory cells were detected and analyzed by flow cytometry (Table 2). This immunological examination showed that memory $CD4^+CD44^+CD62L^+$ and $CD8^+CD44^+CD62L^+$ T-cells were increased after transplantation of MSC and MSC-MV. However, regardless of the expected results (due to anti-inflammatory effects of regulatory T-cells their quantity had to increase) level of $CD4^+CD25^+FoxP3^+$ regulatory cells was dramatically decreased in mouse splenocytes. It might be due to either cell migration to the focus of damage (e.g. a kidney) or other anti-inflammatory mechanisms.

Most of the studies aimed to study CRF are dedicated to autoimmunological and mechanical mechanisms of a renal failure, but toxic immune-related origin of this disease is poorly explored. However, regardless of it, toxic renal failure remains widely spread in human population due to chemotherapy of multiple illnesses such as tuberculosis, cancer, sugar diabetes etc. Moreover, treatment of renal failure also remains less efficient compared to other renal diseases and a long-term perspective always results in a substitution therapy. Advancement in this area would contribute drastically into the improved patients' quality of life and life expectancy.

According to the literature, extracellular vesicles may exert both a positive role in the regeneration process and a negative effect, up to contributing to the development of diseases, causing inflammation, vascular damage and thrombosis. Examining both positive and negative effects on the progression of diseases, including kidney disease, may be a key for organ-preserving and regenerative medicine. Microvesicles derived from mesenchymal stem cells have a pro-regenerative effect in acute renal failure [5].

At this stage of the study, an increased level of regulatory T-cells was recorded in chronic renal failure, that declined during transplantation of both MSCs and microvesicles. At the same time, there is a decrease in the levels of all biochemical blood parameters, as well as albumin in the urine, especially on day 11, which may highlight the onset of the regenerative process. This can also be indicated by increased number of memory T-cells, of both $CD4^+CD44^+CD62L^+$ and $CD8^+CD44^+CD62L^+$ subsets. Regarding T-regulatory cells in the majority of published studies on chronic and acute diseases, Treg depletion exacerbated kidney disease, while their enrichment, on the contrary, increases, which contributes to a high anti-inflammatory effect [11, 13]. There is evidence that certain factors can contribute to both regenerative and toxic effects. In our study, the number of T-regulatory cells not only failed to increase, but significantly decreased on day 11. In published studies on the effect of MSCs on acute renal failure, it was found that a large Treg quantity was accumulated in mouse kidneys and spleens [13]. Thus, in chronic renal failure, it is especially important to study all aspects of the immune response. Based on literature data [7] we also carried out several enzyme-linked immunosorbent assays to study serum level of IFNγ, IL-4, IL-2, IL-10, but no significant changes were observed. Thus, we may

suggest that even chronic renal failure was associated with pro-regenerative effect after inoculation of microvesicles obtained from mesenchymal stem cells that was comparable to that one after MSC transfer. In addition, further examination on both pro-regenerative effect of microvesicles obtained from MSCs, and their effect on T-regulatory and T-memory cells remain to be conducted.

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