Effect of Transplantation of Mesenchymal Stem Cells on Neuronal Survival and Formation of a Glial Scar in the Brain of Rats with Severe Traumatic Brain Injury

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We studied the effect of various methods of transplantation of mesenchymal stem cells on neuronal survival in rat brain 1 and 6 weeks after severe traumatic brain injury. It was found that intracerebral and systemic transplantation of mesenchymal stem cells improves neuronal survival in the piriform cortex of the contralateral hemisphere without affecting neuronal survival in the marginal zone of the traumatic cavity and amygdaloid nuclei. Intracerebral transplantation of mesenchymal stem cells increases the content of the astroglial component of the scar in the borderline zone of the traumatic cavity.

Key Words: *cell therapy; mesenchymal stem cells; traumatic brain injury; glyosis*

Of all stem cells (SC) in adult mammals, mesenchymal stem cells (MSC) located in the bone marrow (BM) stroma are most available for isolation, culturing, and subsequent therapeutic use. The methods of MSC expansion allowing to obtain these cells in amounts sufficient for transplantation were developed [6,15]. It is known that MSC in the absence of artificial inductors can differentiate into osteocytes, adipocytes, and chondrocytes [4,11], while specific factors can induce their differentiation into some other cell lineages, *e.g.* neural cells [10,13]. In light of this, the possibility of using MSC in the therapy of neurodegenerative diseases and traumatic injuries to the nervous system is now intensivily studied [3,8,9,12]. It was shown that cell therapy with MSC can positively affect the morphofunctional recovery of the nervous system due to production of neurotrophic, anti-inflammatory, and antiapoptotic factors by these cells. Thus, MSC transplantation leads to activation of endogenous compensatory mechanisms of nerve tissue regeneration [1,5,7]. At the same time, the effects of MSC transplantation on posttraumatic processes in the brain after severe traumatic brain injury are poorly studied. The questions concerning the limits of MSC application and appropriateness of their transplantation in brain injuries of different severities are not solved yet.

Here we studied the effects of various methods of BM MSC transplantation on neuronal survival in various brain structures in rats with traumatic brain injury. The effects of intracerebral and systemic transplantation of MSC on the formation of the astroglial scar in the marginal zone of the traumatic cavity were also evaluated.

MATERIALS AND METHODS

Experiments were carried out on 4-5-month-old male Wistar–Kyoto rats weighing 180-170 g (n=80). The animals were kept under standard vivarium conditions (12-h illumination regimen and free access to food and water at 23-25°C). The conditions for animal maintenance, surgical manipulations, and postoperation care met the Laboratory Practice Standards in the Russian Federation (2003). The experiments were approved

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by Ethical Committee of the Institute of Experimental Medicine.

Isolation and culturing of MSC. Bone marrow suspension was isolated from the femoral bones immediately after decapitation. The cells were cultured in a monolayer at 37°C and 5% CO_2 (in a CO_2 incubator for 6-7 days in α-MEM (HyClone) containing 20% FCS (Gibco) and 100 μg/ml penicillin/streptomycin (Gibco). Then, the cells were subcultured every 7 days at an initial seeding density of 1.27×10^3 cells/cm^2 using trypsin EDTA (HyClone). The growth medium was replaced every 3 days.

Immunophenotyping of MSC. Rat MSC were immunophenotyped by the method of flow cytofluorometry on an EPICS XL flow cytofluorometer (Beckman Coulter). MSC were stained with antibodies to CD45, CD44, CD90, and CD106 (Beckton Dickinson) according to manufacturer's protocol. Cells of passages 1, 2, and 3 were immunophenotyped. Cells of passages 3 and 4 were used for transplantation.

Modeling traumatic brain injury and cell transplantation. Penetrating trauma of the brain (somatosensory cortex of the left hemisphere) was modeled. The rats were narcotized with zoletil (1 ml/kg). Body temperature during the surgery was maintained at 37°C until the end of narcosis. Animal head was fixed, the skin was cut, the skull was drilled (dura matter was left intact), and trauma was inflicted by the weight-drop method [15] (a 1.3-g weight was dropped from a height of 25 cm in a guide tube). The diameter of damaged area in the neocortex was 2 mm^2, the most pronounced destruction of the nervous tissue was observed at -3.3 mm from bregma. The following brain structures were damaged: the primary and secondary motor cortex and partially somatosensory cortex as well as fragments of the external capsule, corpus callosum, and hippocampus. The operation wound was sutured.

Five experimental groups were formed for the analysis (8 animals per group). Group 1 animals (negative control) received no treatment after modeling of traumatic brain injury. In group 2, the skull was repeatedly opened on day 3 after infliction of brain trauma and 20 μl sterile α-MEM was injected into the marginal zone of the traumatic cavity with a syringe (2×10 μl). In group 3, transplantation of MSC was performed on day 3 after infliction of brain trauma (200,000 cells in 20 μl sterile α-MEM, 2×10 μl). In group 4, systemic transplantation of MSC through the caudal vein was performed on day 3 after infliction of brain trauma (5×10^6 cells in 100 μl α-MEM). In group 5, combined transplantation of MSC (intravenous and into the marginal zone of the traumatic cavity) was performed on day 3 after trauma. Morphological analysis of the brain from rats of each group was carried out 1 and 6 weeks after infliction of brain trauma.

Fixation of the brain. The animals were narcotized and perfused intravitally via the left heart ventricle with 4% paraformaldehyde in PBS (pH 7.2, 10 ml/min perfusion rate, total volume of fixative 200 ml). After decapitation, the brain was removed, and a segment containing the damaged and intact marginal zones was isolated and fixed routinely in paraformaldehyde followed by embedding in paraffin.

Immunohistochemical staining of brain sections. The following markers were detected immunohistochemically: NeuN (marker of neuronal nuclei, Chemicon), glial fibrillary acidic protein (GFAP, marker of astroglia, DAKO). Staining was carried out according manufacturer's protocol with some corrections [2].

Morphometry of the brain. Brain sections were made on a RM2125RT rotation microtome (Leica) producing sections of standard thickness. The number of undamaged, dying, and dead neurons was calculated on sections stained with antibodies to NeuN and poststained with toluidine blue by the method of Nissl in the zone adjacent to the damage (Fig. 1) and in the amygdala of the ipsi- and contralateral hemispheres 1 and 6 weeks after trauma and in the piriform cortex of the ipsi- and contralateral hemispheres 6 weeks after trauma. In each morphological structure, several fields of view were randomly selected (5 in the mar-

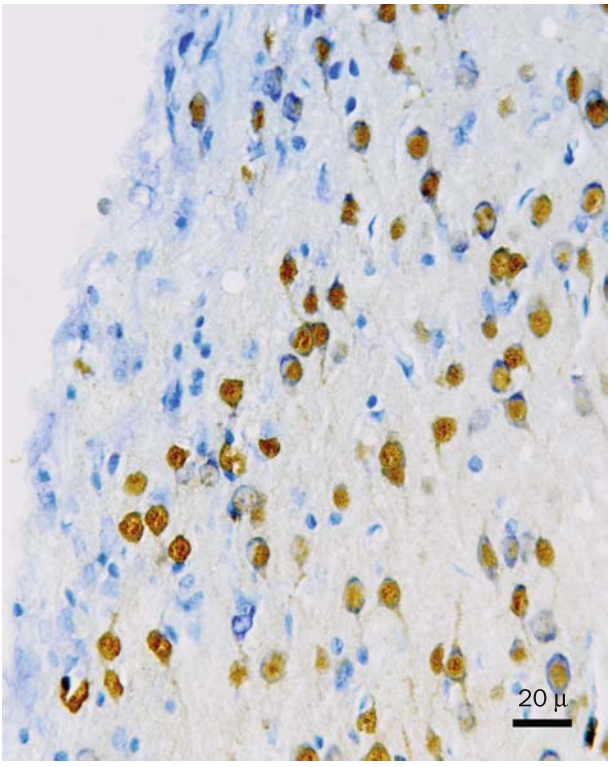

Fig. 1. Visualization of preserved neurons in the marginal zone of traumatic cavity using monoclonal antibodies to NeuN. The group with combined transplantation of MSC 6 weeks after trauma. Here and in Fig. 2: staining with DAB and toluidine blue by the method of Nissl.

ginal zone of the traumatic cavity, 4 in the amygdale, 6 in the piriform cortex) under a microscope (Leica) at ×40 and photographed using a DFC 100 camera (Leica). The thickness of the astroglial scar formed in the marginal zone of the traumatic cavity was evaluated on sections stained with antibodies to GFAP (Fig. 2). A site of the marginal zone was randomly selected under a microscope at ×40 and digital images were made. The thickness of the scar was measured using PhotoM software. The data were processed using Statistica software.

The differences between the groups (in case of normal distribution) were evaluated by ANOVA. The significance of differences between particular groups was evaluated using Tukey's test. If the data distribution does not conform the normal law, the significance of differences between the groups was evaluated by Kruskal–Wallis test and the differences between particular groups were verified using Dunn test.

RESULTS

Immunophenotype of MSC. Cytofluorometric analysis of MSC culture after passage 2 showed that the relative content of $CD45^+$ cells (cells of the hemopoietic lineage) in different animals constituted 3-5% of the total number of cells. $CD44^+$ cells (some hemopoietic cells and a part of MSC population) constituted 10-15%, $CD106^+$ cells (some hemopoietic cells and a part of MSC population) 4-10%, and $CD90^+$ cells (MSC properly) 96-97% of all cells. The cell phenotype determined by surface markers remained unchanged during the first 4 passages.

Results of morphometric study of the brain. The mean numbers of preserved NeuN-immunopositive ($NeuN^+$) neurons in the marginal zone around the traumatic cavity per filed of view at ×40 (3.6 mm^2) 1 and 5 weeks after trauma are presented in Table 1. No significant differences between the experimental groups were revealed.

The mean numbers of $NeuN^+$ neurons in the amygdaloid nuclei in different experimental groups are presented in Table 2. One week after trauma, significant differences were revealed only for the number of preserved neurons in amygdaloid nuclei of the ipsilateral hemisphere in groups 2 and 5. In animals receiving combined transplantation of MSC, the number of preserved neurons in amygdaloid nuclei of the ipsilateral hemisphere (group 5) was higher by 1.7 times than in animals receiving culture medium α-MEM into the marginal zone of the traumatic cavity (group 2). The number of survived neurons in the amygdaloid nuclei tended to be higher after combined administration of MSC (group 5) compared to that in other experimental groups, but the differences were insignificant.

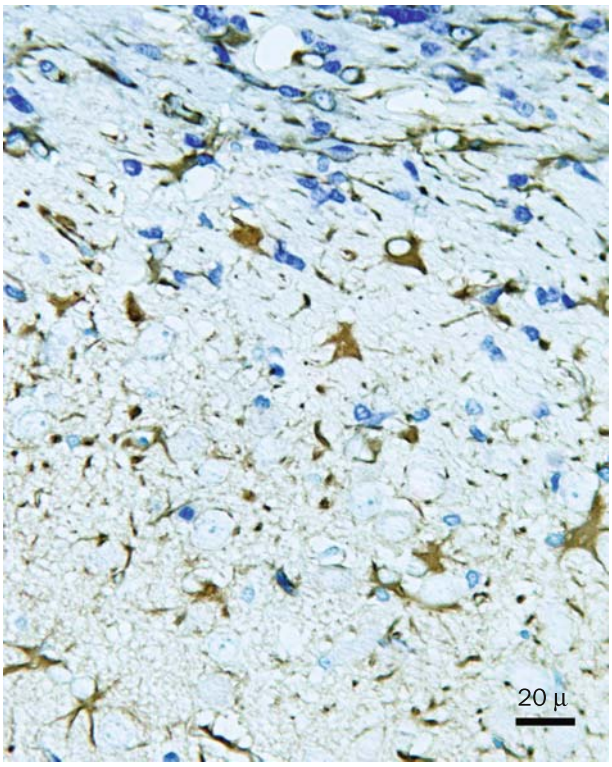

Fig. 2. Visualization of activated astrocytes in the marginal zone of traumatic cavity using monoclonal antibodies to GFAP. The group with combined transplantation of MSC 1 weeks after trauma.

Six weeks after trauma, the number of survived neurons in amygdaloid nuclei of both hemispheres in animals receiving combined MSC transplantation was considerably higher (by 1.5-2 times) than in animals of other experimental groups, including those receiving intravenous or local transplantation of MSC (group 5 and 4, respectively).

TABLE 1. Number of Preserved $NeuN^+$ Neurons in the Marginal Zone of Traumatic Cavity in Animals of Different Experimental Groups at Different Terms of the Experiment (*M*±*SD*)

Group	Time after trauma	
	1 week	6 weeks
Group 1 (negative control)	31.35±8.44	51.28±11.39
Group 2	34.53±12.49	50.23±17.62
Group 3	44.36±16.39	51.61±10.85
Group 4	44.3±9.38	45.1±9.41
Group 5	41.2±16.73	68.1±17.58

Note. Here and in Tables 2, 4: *M*: mean number of preserved neurons per field of view (3.6 mm^2); *SD*: standard deviation.

TABLE 2. Number of Preserved NeuN+ Neurons in Amygdaloid Nuclei in Animals of Different Experimental Groups at Different Terms after Trauma (*M*±*SD*)

Group	Ipsilateral hemisphere		Contralateral hemisphere	
	1 week	6 weeks	1 week	6 weeks
Group 1 (negative control)	26.54±9.13	40.38±15.42	33.38±14.70	41.31±16.56
Group 2	29.63±2.96	32.88±10.59	24.75±8.05	40.95±23.17
Group 3	34.65±13.67	33.12±15	30.57±14.72	31.57±9.69
Group 4	41.13±11.49	22.35±9.59	45.25±8.49	32.35±13.10
Group 5	49.42±19.21	62.13±9.15*	41.25±19.38	63.75±7.34*

Note. Here and in Tables 3, 4: *$p<0.05$.

TABLE 3. Mean Number of Preserved NeuN+ and Dead Hyperchromic Neurons in Piriform Cortex of Ipsilateral and Contralateral Hemispheres in Animals of Different Experimental Groups 6 Weeks after Trauma (*M*±*SD*)

Group		Neurons	
		preserved NeuN+	dead hyperchromic
Group 1 (negative control)	IL	49.67±4.50	3.91±4.05
	CL	44.58±13.50	5±11
Group 2	IL	44.50±3.75	5.5±2.7
	CL	40.50±5.25	5.3±3.4
Group 3	IL	48.67±0.50	1.17±0.75
	CL	53.83±4.00*	2.00±1.05*
Group 4	IL	47.67±3.40	5.33±3.45
	CL	57.5±6.5*	0.83±0.70*
Group 5	IL	53.75±5.70	1.00±0.75
	CL	52.83±5.35*	0.6±0.3*

Note. IL: ipsilateral hemisphere; CL: contralateral hemisphere. The data are presented as median±median confidence interval.

The mean number of morphologically unchanged NeuN+, dying and dead hyperchromic (by the result of toluidine blue staining after Nissl) neurons in the piriform cortex of both ipsilateral and contralateral hemispheres in animals of different experimental groups 6 weeks after trauma are presented in Table 3. It was shown that transplantation of MSC irrespective of the used method by 1.2-1.3 times increased the number of morphologically preserved NeuN+ neurons in the piriform cortex of the contralateral hemisphere and considerably decreased the number of hyperchromic nerve cells in this area.

TABLE 4. Mean Width of Astroglial Scars (mm) in Marginal Zone around the Traumatic Cavity in Animals of Different Experimental Groups 6 Weeks after Trauma

Group	*M*±*SD*
Group 1 (negative control)	17.35±4.23
Group 2 (intracerebral injection of α-MEM culture medium)	17.40±3.86
Group 3 (intracerebral MSC transplantation)	36.26±3.57*
Group 4 (intravenous MSC transplantation)	63.22±3.57
Group 5 (combined MSC transplantation)	18.12±3.86

Note. Data are presented at objective magnification ×40.

The mean widths of the astroglial scars formed in the marginal zone around the traumatic cavity 6 weeks after trauma in animals of all experimental groups are presented in Table 4. In group 2 animals, the scar was 2-fold wider than in other experimental groups. No significant differences between the groups were revealed.

Our findings suggest that MSC transplantation irrespective of the used method produces only limited positive effect in severe brain injury involving not only the cortex, but also subcortical structures. This effect consisted in preservation of neurons in the piriform cortex of the contralateral hemisphere damaged as a result of brain strike against skull base bones at the moment of trauma. Combined MSC transplantation also protects neurons in amygdaloid nuclei of both hemispheres. Intracerebral MSC transplantation into the marginal zone of traumatic cavity aggravates trauma aftereffects, which is seen from widening of the astroglial scar around the traumatic cavity.

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