Neuroscience and Behavioral Physiology, Vol. 43, No. 5, June, 2013

Effects of Transplantation of Human Cord Blood Mononuclear Cells Expressing the Recombinant *VEGF* and *FGF2* Genes into Spinal Cord Traumatic Injury Sites in Rats

G. F. Shaimardanova,[1] Ya. O. Mukhamedshina,[2] A. A. Rizvanov,[3,4,5] I. I. Salafutdinov,[4,5] and Yu. A. Chelyshev[2]

UDC 616.832-001-089.843:611.018.5:599.323.4

Translated from Morfologiya, Vol. 142, No. 4, pp. 31–36, July–August, 2012. Original article submitted February 14, 2011. Revised version received March 12, 2012.

A model of dosed T_{VIII} spinal cord contusion trauma in rats was used to study the effects of immediate single-dose transplantation of human cord blood mononuclear cells transformed with the recombinant genes for neurotrophic factors – vascular endothelia growth factor (VEGF) and fibroblast growth factor 2 (FGF2) – into the injury zone. A further group of animals, in the same conditions, received the same cells transfected with plasmid pEGFP-N2. EGFP-labeled cells were detected in the white matter for 21 days after transplantation at distances of at least 10 mm in the rostral and caudal directions from the administration point. By 30 days after transplantation with cells transfected with plasmid pBud-VEGF-FGF2, the area of intact gray matter 3 mm from the trauma epicenter increased by more than 60%. By this time, the outer areas of the white matter in animals of this group, 1.5 cm from the trauma epicenter, showed an average 30% increase in the number of perivascular cells expressing platelet-derived growth factor β receptors (PDGFβR). Addition of therapeutic genes VEGF an FGF2 to the trauma injury zone and their expression in carrier cells stimulated vascularization and post-traumatic regeneration of the spinal cord.

Keywords: spinal cord, regeneration, cord blood cells, VEGF, FGF2.

Delivery of therapeutic genes to injured areas constitutes a potential approach to stimulating neuroregeneration. Various differentiated, stem, and induced pluripotent stem and progenitor cells are used for this method [1, 4, 13]. Cord blood cells have significant potential, because of their low immunogenicity, availability, simplicity and safety of use, and ability to survive prolonged storage. These cells have been subjected to intensive study as a source of stem and progenitor cells for transplantation in cerebral traumas and ischemia, as well as for the treatment of neurodegenerative diseases [12, 15]. Studies of the efficacy of delivering neurotrophic factor genes using cell carriers have only started recently. We selected a combination of the vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF2) for stimulation of the post-traumatic regeneration of the spinal cord. Both molecular determinants are members of the neurotrophic factors family, while *VEGF* promotes neovascularization. The effects of this combination of genes on neuroregeneration processes have not been studied. We therefore constructed, for the first

[1] Laboratory for the Molecular Basis of Pathogenesis (Director: Professor V. M. Chernov), Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, a/ya 30, 420111 Kazan; e-mail: gulnara_kzn@rambler.ru.

[2] Department of Histology, Cytology, and Embryology (Director: Professor Yu. A. Chelyshev), Kazan State Medical University, Kazan; e-mail: Yanakazmedhist1@rambler.ru, chelyshev-kzn@yandex.ru.

[3] Central Research Laboratory (Director: S. R. Abdulkhanov), Kazan State Medical University, Kazan; e-mail: rizvanov@gmail.com.

[4] Department of Genetics (Director: Professor B. I. Barabanshchikov), Kazan (Volga) Federal University, Kazan; e-mail: sal.ilnur@gmail.com.

[5] Republican Clinical Hospital, Ministry of Health of the Republic of Tatarstan, Kazan.

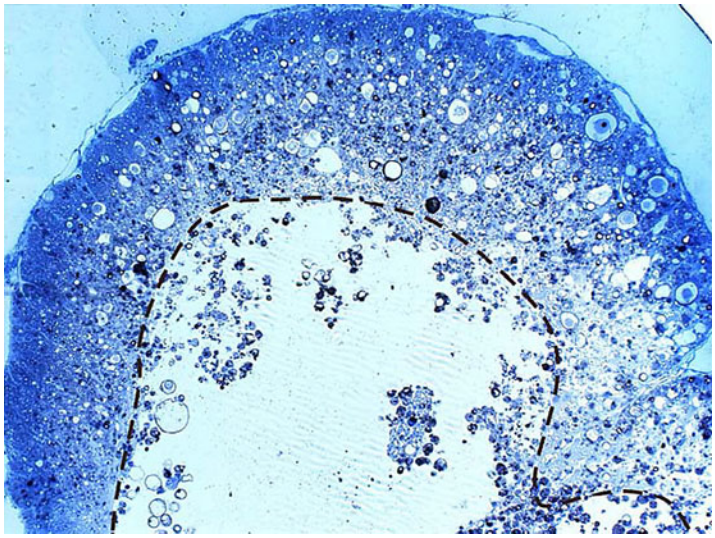

Fig. 1. Fragment of rat spinal cord 3 mm from the epicenter 30 days after transplantation of human cord blood mononuclear cells transfected with plasmid pBud-EGFP into the contusional injury area at the T_{VIII} level. The dotted line shows the boundary between the central pathological cavity and the zone of relatively intact white matter able to recover. Magnification ×40.

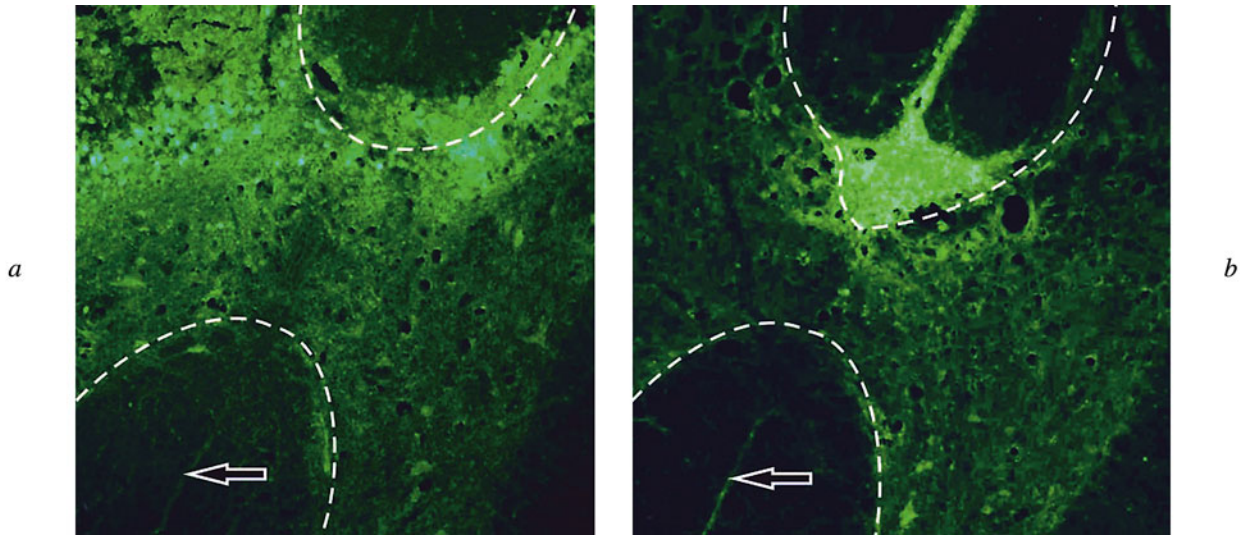

Fig. 2. Intense specific staining for EGFP in human cord blood mononuclear cells transfected into the spinal cord injury site in the posterior fasciculi at the boundary with the gray matter (*a*) and local staining in the posterior corticospinal tract and along the posterior medical sulcus (*b*). *a*) 7 days; *b*) 14 days after trauma. Dotted lines show boundaries between gray and white matter; arrows how the anterior median cleft. Magnification ×250.

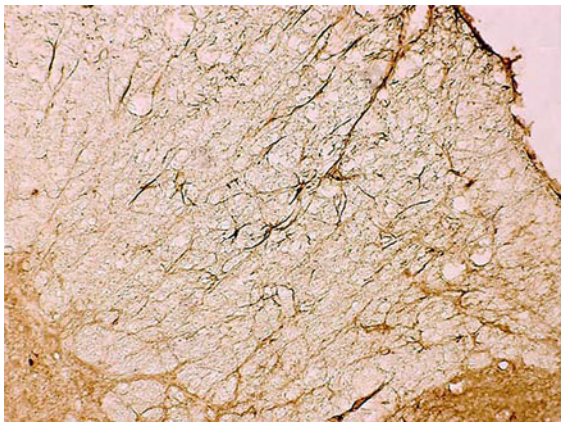

Fig. 4. Expression of PDGFβR in zone 3 of the white matter of the rat spinal cord 1.5 cm from the trauma epicenter in the rostral direction 14 days after transplantation of human cord blood cells transfected with plasmid pBud-EGFP. Immunocytochemical reaction; magnification ×250.

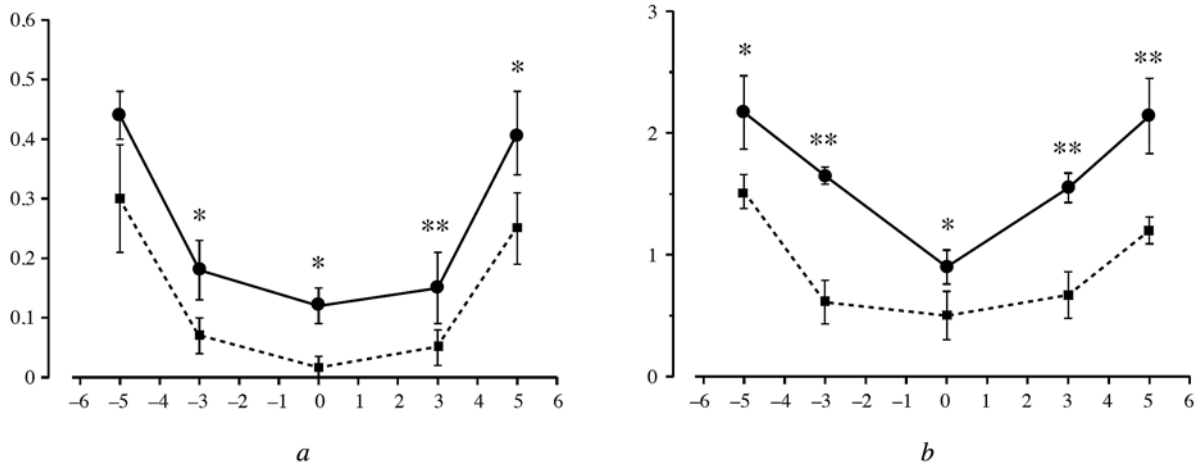

Fig. 3. Area of preserved gray (*a*) and white (*b*) matter on transverse sections of rat spinal cord at the level of T_{VIII} 30 days after transplantation of human cord blood cells transfected with plasmid pEGFP-N2 (dotted line) and plasmid pBud-VEGF-FGF2 (continuous line) into the contusion injury zone. Abscissas show distance from the trauma epicenter – from 0 in the caudal (at left) and rostral directions (at right) (mm); ordinates show areas (mm^2). Significant differences: $*p < 0.05$; $**p < 0.01$.

time, a double-cassette plasmid, pBud-VEGF-FGF2, which simultaneously expresses both genes [3]. This was used to transfect human cord blood mononuclear cells [12]. The aim of the present work was to use a model of dosed contusion trauma to the rat spinal cord to study the effects of immediate single deliveries of human cord blood mononuclear cells genetically modified with the plasmid vector expressing two recombinant human neurotrophic factor genes, VEGF and FGF2, to the injury site.

Materials and Methods

Experiments were performed on 25 white laboratory rats, females and males, weighing 200–250 g. Laboratory animals were kept and used in compliance with the regulations applying at the Kazan State Medical University and approved by the Ethics Committee. Animals were kept in standard conditions with free access to water and feed. Rats were anesthetized by i.p. injection of chloral hydrate (Sigma, USA) (80 mg/ml, 0.4 ml/100 g). Animals underwent laminectomy at the level of T_{VIII}. Dosed contusion trauma to the spinal cord was applied as described previously [2]. Collection of human cord blood and extraction of the mononuclear cell fraction were performed as described previously [12]. Extracted cells were transfected by electroporation [12] with plasmids pBud-VEGF-FGF2 [3] for animals of group 1 (n = 7). Immediately after imposition of trauma, transfected cells (10^6 cells in 5 µl of DPBS (phosphate-buffered saline, sterile, lacking Ca^{2+} and Mg^{2+} ions, BioloT, Russia)) were injected at two points 1 mm rostral and caudal to the trauma epicenter and 0.5 mm lateral to the midline using a Hamilton syringe (Sigma, USA). In the same conditions, animals of group 2 (n = 18) received the same quantity of analogous cells transfected with plasmid pBud-EGFP-N2 (Clontech, USA), carrying the gene for enhanced green fluorescent protein (EGFP). During the seven days after surgery, all animals received i.m. gentamicin (5 mg/kg) once daily. Animals of group 1 at 30 days and animals of group 2 at 2, 4, 7, 14, 21, and 30 days after trauma were anesthetized and subjected to transcardiac perfusion of 4% paraformaldehyde solution (4°C). Fragments of spinal cord of length 5 cm were collected along with vertebrae. After 12 h of fixation, spinal cord was collected and divided into five equal parts.

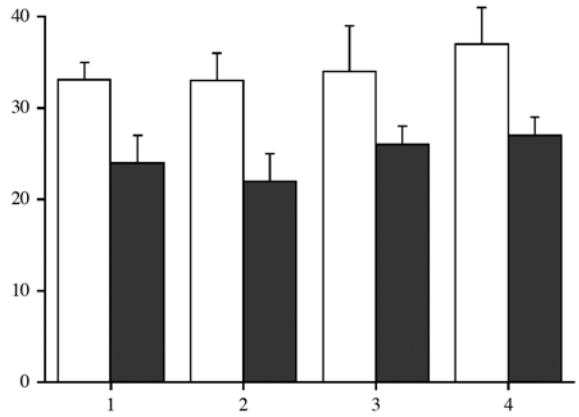

Fig. 5. Numbers of PDGFβR+ cells in different zones of the white matter 30 days after surgery. White columns show animals of group 1 (pBud-VEGF-FGF2); dark columns show animals of group 2 (pBud-EGFP-N2). The abscissa shows morphometric zones; the ordinate shows cell numbers. Vertical bars show mean square deviations; differences between control and experimental values significant at $p < 0.05$.

The survival and migratory potential of cells transfected with plasmid pBud-EGFP-N2 were studied in cryostat

transverse spinal cord sections. Semithin sections stained with methylene blue prepared from specimens collected 30 days after surgery and embedded in Epon-Araldite were used for measurement of the areas of intact gray and white matter at distances of 3 and 5 mm from the trauma epicenter in the rostral and caudal directions.

Perivascular cell detection was performed on cryostat transverse sections of the spinal cord at a distance of 1.5 cm from the trauma epicenter by an indirect immunoperoxidase method using antibodies to platelet growth factor β receptors (PDGFβR) (Sigma, USA) at a dilution of 1:100. PDGFβR$^+$ cells were counted in digital images in four defined zones of the white matter: 1 – the ventromedial part of the anterior fasciculus, adjacent to the median fissure on the right side; 2 – the same but on the left side; 3 – the lateral part of the lateral fasciculus in the frontal plane passing through the central canal, right side; 4 – the same but on the left side [1]. Specimens were examined and digital images were obtained using an Axio Image A1 microscope (Carl Zeiss, Germany). Morphometric results were processed using Student's *t* test.

Results

In the area of spinal cord contusions, during the early post-trauma period (day 7), the relatively preserved white matter showed edema, degeneration of nerve fibers rostral and caudal to the trauma epicenter, and destructive changes to their myelin sheaths. The greatest level of damage affected the gray matter, where chromatolysis and neuron death were seen. In animals given plasmid pBud-EGFP-N2, by day 30, the boundaries between the gray and white matter were not visible in the trauma area or at distances of 3 and 5 mm in the caudal and rostral directions. Cross sections showed a central zone of completely disintegrated tissue, apparent as an extensive cavity containing fragments of degenerated cells (Fig. 1). The size of this cavity decreased with increasing distance from the trauma epicenter: at a distance of 3 mm, it was two thirds and at 5 mm it was one half of the area of the section. At the periphery of the sections, the structure of the white matter was retained and signs of damage were less marked. At this location there was an abundance of microcavities separated by layers of connective tissue, along with signs of astrogliosis.

Clear differences in the structure of the trauma epicenter in animals given plasmids pBud-VEGF-FGF2 (group 1) and rats of group 2 were not seen.

The spinal cord of animals of group 2, given cord blood mononuclear cells transfected with plasmid pEGFP-N2, showed specific staining at distances of up to 10 mm in the rostral or caudal directions from the injection point. This staining was most intense in the early periods after trauma and administration of cells (days 2–6) and was clearly present at later periods (days 14–21) (Fig. 2), gradually decreasing to day 30. EGFP-labeled cells were present in surviving gray and white matter tissue and entered the pathological cavity. While two days after transplantation, fluorescent cells were distributed more uniformly and mainly in the white matter, they formed accumulations with intense fluorescence by six days both in the ventral parts of the posterior fasciculi and, particularly, in the adjacent dorsal part of the gray matter (see Fig. 2, *a*). By day 7, the area with characteristic staining for EGFP occupied a larger volume and staining intensity increased, possibly resulting from increased EGFP expression in transplanted cells or increases in the number of such cells due to active proliferation. By day 14, the area containing EGFP$^+$ cells decreased significantly. By this time, specific EGFP fluorescence was detected in compact groups of cells, mainly in the ventral part of the posterior fasciculi at the boundary with the gray matter (see Fig. 2, *b*).

By day 30 after transplantation of cells transfected with plasmid pBud-VEGF-FGF2 (group 1), the area of surviving gray matter increased by 66% 3 mm in the rostral direction from the trauma epicenter and by 61% in the caudal direction (Fig. 3, *a*). Comparison of groups 1 (pBud-VEGF-FGF2) and 2 (pBud-EGFP-N2) showed that differences in the areas of surviving gray matter 5 mm from the epicenter were less marked, though, as at 3 mm from the epicenter, values were 40% greater in the rostral direction and 31% greater in the caudal direction ($p < 0.05$) (see Fig. 3, *a*).

A decrease in the area of tissue destruction on transplantation of cells transfected with plasmid pBud-VEGF-FGF2 was also seen in the white matter (see Fig. 3, *b*). By experimental day 30, 3 mm in the rostral and caudal directions from the trauma epicenter, the areas of surviving white matter in animals of group 1 were 56% and 63% greater than those in the same directions in group 2. At 5 mm in the rostral and caudal directions from the trauma epicenter, the areas of surviving white matter in animals of group 1 increased by 45% and 43%, respectively ($p < 0.05$) (see Fig. 3, *b*). Morphometric results provided evidence that the supporting influence on gray and white matter survival resulting from delivery of therapeutic genes dropped off with increasing distance from the trauma epicenter and cell administration points.

Precipitate of the end product of the immunohistochemical reaction with anti-PDGFβR antibodies was seen predominantly in the white matter in cells associated with blood vessels. The long PDGFβR-immunopositive processes of these perivascular cells were oriented along blood vessels passing in the radial direction (Fig. 4). The largest numbers of PDGFβR$^+$-cells were seen in the outer zones of the white matter. No differences were seen in the distribution pattern or immunohistochemical reaction precipitate intensity in specimens obtained from animals of groups 1 (pBud-VEGF-FGF2) and 2 (pBud-EGFP-N2). In animals of group 1, the number of perivascular cells expressing PDGFβR in the outer zones of the white matter 1.5 cm from the trauma epicenter increased by an average of 30% (Fig. 5).

Discussion

The supporting influence of transplanted cells and the regeneration stimulators delivered by them on recovery of lost

spinal cord functions depend to a significant extent on the survival and duration of these cells in the recipient tissue, as well as on their migratory potential. In this regard, our data are consistent with results reported elsewhere [5, 14], in which genetically modified stem mesenchymal cells transplanted into the intact rat spinal cord, in the presence of adequate immunosuppressive therapy, not only survived for at least three weeks, but also expressed the transgene. Increases in specific staining for EGFP in the early post-transplantation period in the spinal cord trauma zone in rats, after delivery by human cord blood cells, may result from the proliferation of these cells and/or increases in their expression of the transgene.

In our experimental conditions, the prolonged expression of EGFP given, without immunosuppression, at the moment of trauma rather than after the peak of the cytostatic actions of proinflammatory cytokines and cellular immune responses led to expression not only of the high survival ability of the transplanted cells, but also of the potential for maintaining the expression of therapeutic genes.

It can be suggested that transfection of cells with neurotrophic factor genes in the combination used here, VEGF and FGF2, increases the number of viable cells and prolongs their persistence at the trauma site. This possibility has been demonstrated for neural stem cells transfected with the VEGF gene when transplanted into spinal cord contusion trauma zones [9].

The importance of the migratory potential of human cord blood cells after transplantation into spinal cord trauma sites identified here should be noted. The migratory potential of the cord blood mononuclear cells studied here in the spinal cord contusion zone was greater than or at the level of typical hematopoietic and neural stem cells in analogous experiments [7, 10].

PDGF-B binds to PDGFβR receptors, stimulating the differentiation of pericytes from PDGFβR-expressing precursors, resulting in a supportive influence on the vascularization process [6, 8]. Impairments to this are demonstrated in PDGF-B and PDGFβR defects [11]. The increase in the number of PDGFβR$^+$ cells at 30 days seen in the present experiments reflects an increase in vascularization of the white matter around the traumatic spinal cord injury. This effect appears to result from the action of VEGF produced by the transplanted cord blood cells.

Our results provide evidence that delivery of the therapeutic VEGF and FGF2 genes using human cord blood mononuclear cells leads to decreases in the areas of destruction of the gray and white matter, stimulates vascularization of brain tissue, and supports the post-traumatic regeneration of brain tissue.

This study was supported by a State Contract from the Federal Targeted Program of the Russian Federation Ministry of Education and Sciences, No. 16.512.112101.

REFERENCES

1. S. V. Lebedev, A. V. Karasev, V. P. Chikhonin, et al., "Studies of the efficacy of human neural stem cell transplantation into rats with spinal cord trauma: use of functioning loading tests and the BBB method," *Byull. Eksperim. Biol.*, **149**, No. 3, 355–360 (2010).
2. S. V. Lebedev, S. V. Timofeev, A. V. Zharkov, et al., "Loading tests and the BBB method in assessment of motor impairments after contusional trauma to the spinal cord," *Byull. Eksperim. Biol.*, **145**, No. 10, 471–476 (2008).
3. I. I. Salafutdinov, A. K. Shafigullina, M. E. Yalvach, et al., "Effects of once-off expression of different isoforms of vascular endothelial growth factor VEGF and basic fibroblast growth factor FGF2 on the proliferation of human umbilical vein blood endothelial cells HUVEC," *Klet. Transpl. Tkan. Inzh.*, **5**, No. 2, 62–67 (2010).
4. Yu. A. Chelyshev and I. V. Viktorov, "Cellular techniques for remyelination in spinal cord trauma," *Nevrol. Vestn. Bekhtereva*, No. 1, 49–55 (2009).
5. L. Bai, D. P. Lennon, V. Eaton, et al., "Human bone marrow-derived mesenchymal stem cells induce Th-2-polarized immune response and promote endogenous repair in animal models of multiple sclerosis," *Glia*, **57**, No. 11, 1192–1203 (2009).
6. C. Betsholtz, P. Lindblom, and H. Gerhardt, "Role of pericytes in vascular morphogenesis," *EXS*, **94**, 115–125 (2005).
7. S. S. Han, Y. Liu, C. Tyler-Polsz, et al., "Transplantation of glial-restricted precursor cells into the adult spinal cord: survival, glial-specific differentiation, and preferential migration in white matter," *Glia*, **45**, 1–16 (2004).
8. M. Hellstrom, H. Gerhardt, M. Kalen, et al., "Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis," *J. Cell Biol.*, **153**, 543–553 (2001).
9. J. H. Lin, W. A. Pennant, H. M. Lee, et al., "Neural stem cells modified by a hypoxia-inducible VEGF gene expression system improve cell viability under hypoxic conditions and spinal cord injury," *Spine*, **36**, No. 11, 857–864 (2011).
10. A. R. Khalatbary and T. Tiraihi, "Localization of bone marrow stromal cells in injured spinal cord treated by intravenous route depends on the hemorrhagic lesions in traumatized spinal tissue," *Neurol. Res.*, **29**, No. 1, 21–26 (2007).
11. P. Lindahl, B. Johansson, P. Leveen, and C. Betsholtz, "Pericyte loss and microaneurysm formation in PDGF-B deficient mice," *Science*, **277**, 242–245 (1997).
12. A. A. Rizvanov, A. P. Kiyasov, I. M. Gaziziov, et al., "Human umbilical cord blood cells transfected with VEGF and L(1)CAM do not differentiate into neurons but transform into vascular endothelial cells and secrete neurotrophic factors to support neurogenesis – a novel approach in stem cell therapy," *Neurochem. Int.*, **53**, No. 6–8, 389–394 (2008).
13. M. Ronaghi, S. Erceg, V. Moreno-Manzano, and M. Stojkovic, "Challenges of stem cell therapy for spinal cord injury: human embryonic stem cells, endogenous stem cells, or induced pluripotent stem cells?" *Stem Cells*, **28**, No. 1, 93–99 (2010).
14. M. W. Ronsyn, Z. N. Berneman, V. F. Van Tendeloo, et al., "Can cell therapy heal a spinal cord injury?" *Spinal Cord*, **46**, No. 8, 532–539 (2008).
15. Z. M. Zhao, H. J. Li, S. H. Lu, et al., "Intraspinal transplantation of CD34+ human umbilical cord blood cells after spinal cord hemisection injury improves functional recovery in adult rats," *Cell Transplant.*, **13**, No. 2, 113–122 (2004).