Multipotent Mesenchymal Stromal Cells of Autologous Bone Marrow Stimulate Neoangiogenesis, Restore Microcirculation, and Promote Healing of Indolent Ulcers of the Stomach

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Translated from *Kletochnye Tehnologii v Biologii i Medicine*, No. 4, pp. 195-200, August, 2008
Original article submitted April 28, 2008.

The effects of precultured multipotent mesenchymal stromal cells from autologous bone marrow on microcirculation and regeneration of experimental indolent stomach ulcer were studied in experiments on rats. It was demonstrated that precultured multipotent mesenchymal stromal bone marrow cells delivered to the zone of chronic gastric ulcer stimulate neoangiogemesis, improve microcirculation in the gastric mucosa, and promote ulcer healing.

Key Words: *bone marrow; microcirculation; experimental ulcer*

Ulcer disease of the stomach and duodenum develops as a result of impaired reparative regeneration in the mucosa caused by microcirculatory disturbances in the lamina propria and submucosa [2,4]. Microcirculatory disturbances are associated with inhibition of migration of endothelial cell precursors into the zone of ulcer defect. These cells are of mesenchymal origin and are produced by multipotent mesenchymal stromal cells (MMSC) of the bone marrow [3,7,8,12,13].

Endotheliocyte precursors similarly to bone marrow MMSC are low-differentiated cells intensively secreting regulatory peptides, immunocytokines, and growth factors [12] producing proangiogenetic and morphogenetic effects. Chronic gastric ulcers are associated with deficiency of these factors [9].

Here we used cultured MMSC from autologous bone marrow for stimulation of angiogenesis, improvement of microcirculation, and effective regeneration of non-healing autoimmune gastric ulcers (NHGU).

MATERIALS AND METHODS

NHGU were modeled in male Wistar rats (body weight 250-300 g). The animals received 1.5 g homogenized gastric mucosa from an allogeneic rat in a volume of 1.5-2.0 ml physiological saline with 1.5 ml Freund adjuvant. The homogenate was injected subcutaneously into animal paw 3 times with 7-day intervals. After 35-40 days, a chronic gastric ulcer with a diameter of 5-7 mm developed. The ulcer had uneven edges and was surrounded by the zone of pronounced edema, hyperemia, and small erosions. No tendency to ulcer healing was observed within 2.5 months. The animals were divided into 2 groups: group 1 (experiment, n=15) included rats with chronic NHGU receiving transplantation of autologous bone marrow MMSC, group 2 (control, n=15) comprised rats with chronic NHGU receiving physiological saline. Bone marrow cells were obtained from the iliac bones under ether narcosis on days 30-35 after NHGU modeling. The

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0007-4888/08/1464-0512

bone cavity was washed with 0.5 ml buffered physiological saline. For isolation of MMSC, the bone marrow cells were cultured *in vitro* in IMDM (Gibco) containing 10% bovine serum (HyClone) on Perti dishes at 37°C, 5% CO_2, and 95% humidity. MMSC monolayer was attained after 9-12 days. Bone marrow cells were cultured not only for increasing cell mass, but also for improving their bioregulatory and functional activity.

On day 45 after NHGU modeling, laparotomy and gastrotomy were performed, and precultured bone marrow MMSC (4 mln. cells suspended in 0.5 ml NaCl) were injected into 4 equally-spaced points around the ulcer; control animals received 0.5 ml 0.9% NaCl.

The efficiency of cell therapy was evaluated visually and by planimetric, morphometric (blood vessel count per field of view) [1], and morphological methods on days 10, 20, and 30 after cell transplantation. For morphological study, the stomach was fixed in 10% neutral formalin, 5-μ paraffin sections were stained with hematoxylin and eosin.

For quantitative evaluation of NHGU regeneration, we also studied microcirculatory bed of the gastric mucosa in the zone of ulcer defect in dynamics. The analysis was performed by the method of laser Doppler flowmetry (LDF) using a LAKK-02 laser capillary blood flow analyzer (Lazma) [5] approved by Ministry of Health of Russian Federation for the use in clinical practice, Protocol No. 1, 13.01.1993, Committee for Clinical Diagnostic Devices [6]). This analyzer recorders parameter of microcirculation (PM) in 1-1.5 mm^3 tissue, which is a function of erythrocyte concentration in the analyzed tissue volume and their averaged rate; PM is measured in relative perfusion units (PU) [5]. At this stage, some additional parameters of microcirculation were calculated: arithmetic mean of PM (M); mean square deviation from the mean M (σ) for blood flow amplitude; and coefficient of variation (CV) calculated by the formula $\sigma/M\times100\%$. We also performed computer analysis of perfusion fluctuation in the zone of the ulcer during simulation of myogenic activity of vasomotors, neurogenic influences inducing blood flow changes, and passive modulation of blood flow. Measurement of these parameters yielded an integral characteristics of hemodynamic, a fluxmotion index (FMI) calculated by the formula:

$$FMI=A_{maxLF}/(A_{maxHF}+A_{maxCF}),$$

where A_{maxLF}, A_{maxHF}, and A_{maxCF} are maximum amplitudes of blood flow fluctuations in ranges of 3-12, 12-24, and 50-90 min^{-1}, respectively [5].

The results were processed using Student's t test.

RESULTS

On day 10 after MMSC transplantation, we observed a significant decrease in ulcer defect area compared to the control (120±31 mm^2 and 246±52 mm^2, respectively). Under the effect of MMSC transplantation, the area of the ulcer defect continued to decrease after 20 days and on day 30 it was 9±2 mm^2 (*vs.* 53.0±4.1 mm^2 in the control group). These findings suggest that MMSC stimulate the process of active ulcer regeneration.

For evaluation of the relationship between regeneration processes and the state of the microcirculatory bed, we performed a dynamic study of PM in the zone of the ulcer defect, in the marginal zone, and at a distance of 0.5 cm from the edge of the ulcer. These studies were preceded by evaluation of PM in the antral gastric mucosa of intact rats (typical location of modeled ulcers). These results served as the control in evaluation of the state of microcirculatory bed in spontaneous regeneration of NHGU and in the dynamics of their healing after MMSC transplantation. The mean parameters of microcirculation in the antrum are presented in Table 1.

Native LDF of groups 1 and 2 animals in the zone of ulcer on days 10 and 30 of treatment is presented (Fig. 1). On day 10, PM in animals receiving bone marrow MMSC was 2-fold higher than in controls. On day 30, PM also increased in ex-

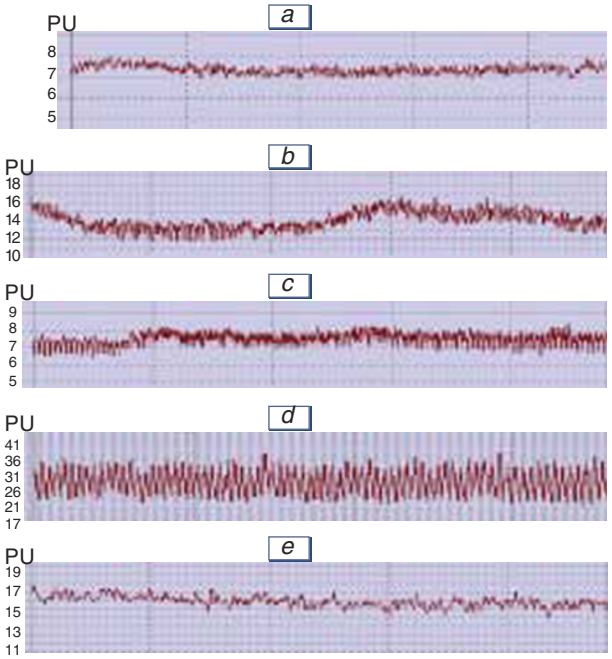

Fig. 1. LDF of gastric mucosa in the zone of ulcer. *a*) initial; *b*) on day 10 after transplantation of bone marrow MMSC; *c*) on day 10 after injection of physiological saline (control); *d*) on day 30 after transplantation of bone marrow MMSC; *e*) on day 30 after injection of physiological saline (control).

TABLE 1. Parameters of Microcirculation in the Zone of NHGU in the Dynamics of Reparative Process in Rats Receiving and Not Receiving Transplantation of Bone Marrow MMSC (PU, *M*±*m*)

Time of observation, day			PM	σ	CV	FMI
Normal values			43.2±19.0	5.39±0.20	4.35±3.00	0.74±0.30
Initial	ulcer		2.13±0.50	8.78±3.20	412.9±43.2	0.14±0.04
	ulcer edge		1.88±0.40	3.58±0.20	190.3±6.8	0.10±0.01
	0.5 cm from ulcer edge		4.15±2.10	19.0±11.2	457±38	0.8±0.3
day 10	ulcer	control	2.43±0.2	0.20±0.04	420.0±39.6	0.80±0.57
		experiment	3.21±2.30	0.94±0.50	29.2±21.0	0.30±0.07
	ulcer edge	control	1.44±0.10	0.90±0.39	62.7±18.0	0.70±0.18
		experiment	7.19±4.60	2.52±0.20	34.9±11.0	0.70±0.23
	0.5 cm from ulcer edge	control	6.35±0.30	1.80±0.41	343.0±37.2	1.10±0.33
		experiment	7.78±4.80	6.38±0.20	81.9±6.7	0.80±0.07
day 20	ulcer	control	1.88±0.40	3.58±0.40	190.0±47.5	0.30±0.06
		experiment	10.1±4.7	4.04±0.20	23.5±10.0	0.60±0.21
	ulcer edge	control	5.89±0.60	5.42±0.30	92.0±22.7	1.40±0.15
		experiment	11.8±5.2	5.51±0.40	46.6±22.0	2.80±0.04*
	0.5 cm from ulcer edge	control	9.86±4.90	26.1±17.9	263.7±34.2	0.25±0.06
		experiment	15.5±2.0*	1.00±0.11	2.88±0.31	0.94±0.63
day 30	ulcer	control	3.99±3.70	5.47±0.20	137±38	0.30±0.06
		experiment	12.2±6.9	9.35±4.60	76.4±34.0	1.50±0.17
	ulcer edge	control	8.31±4.10	9.9±4.4	119.0±27.6	1.00±0.12
		experiment	25.2±16.0	1.59±0.10	14.2±7.8	1.70±0.21
	0.5 cm from ulcer edge	control	14.7±7.2	14.6±6.9	99.8±48.2	0.88±0.57
		experiment	26.9±3.9*	3.0±2.1	11.0±5.2	1.73±0.12*

Note. *p<0.05 compared to day 10.

perimental animals, amplitude characteristics of microcirculation were more pronounced than in the control; PM in controls also increased and approached the values observed in the experimental group of day 10.

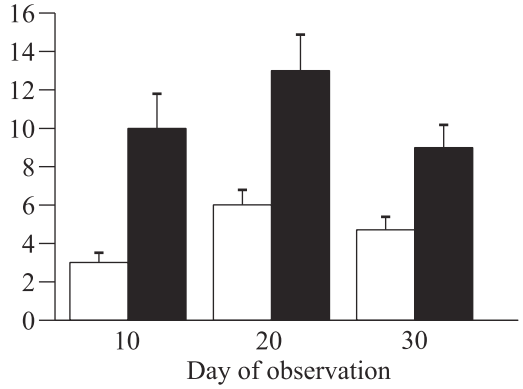

Fig. 2. Dynamics of the mean number of blood vessels per field of view in the marginal zone of the ulcer defect of non-healing autoimmune gastric ulcers. Ordinate: number of vessels per field of view in the marginal zone of the ulcer defect. Open bars: control; dark bars: bone marrow MMSC. *p<0.05 compared to the control.

Evaluation of the integral characteristics of hemodynamics showed that not only PM, but also FMI significantly increased starting from day 20 in both groups (Table 1), which attested to improvement of the microcirculatory bed and recovery of its adequate regulation in response to simulated loads.

Morphometry of vessels in the zone of the ulcer defect in the dynamics of NHGU healing also confirmed recovery of microcirculation (Fig. 2). Injection of MMSC considerably increased the number of microvessels per field of view compared to the corresponding parameter in the control group, which attested to stimulation of neoangiogenesis.

The positive effect of bone marrow MMSC on neoangiogenesis and regulation of microcirculation is also seen from activation of reparative regeneration confirmed by the results of morphological

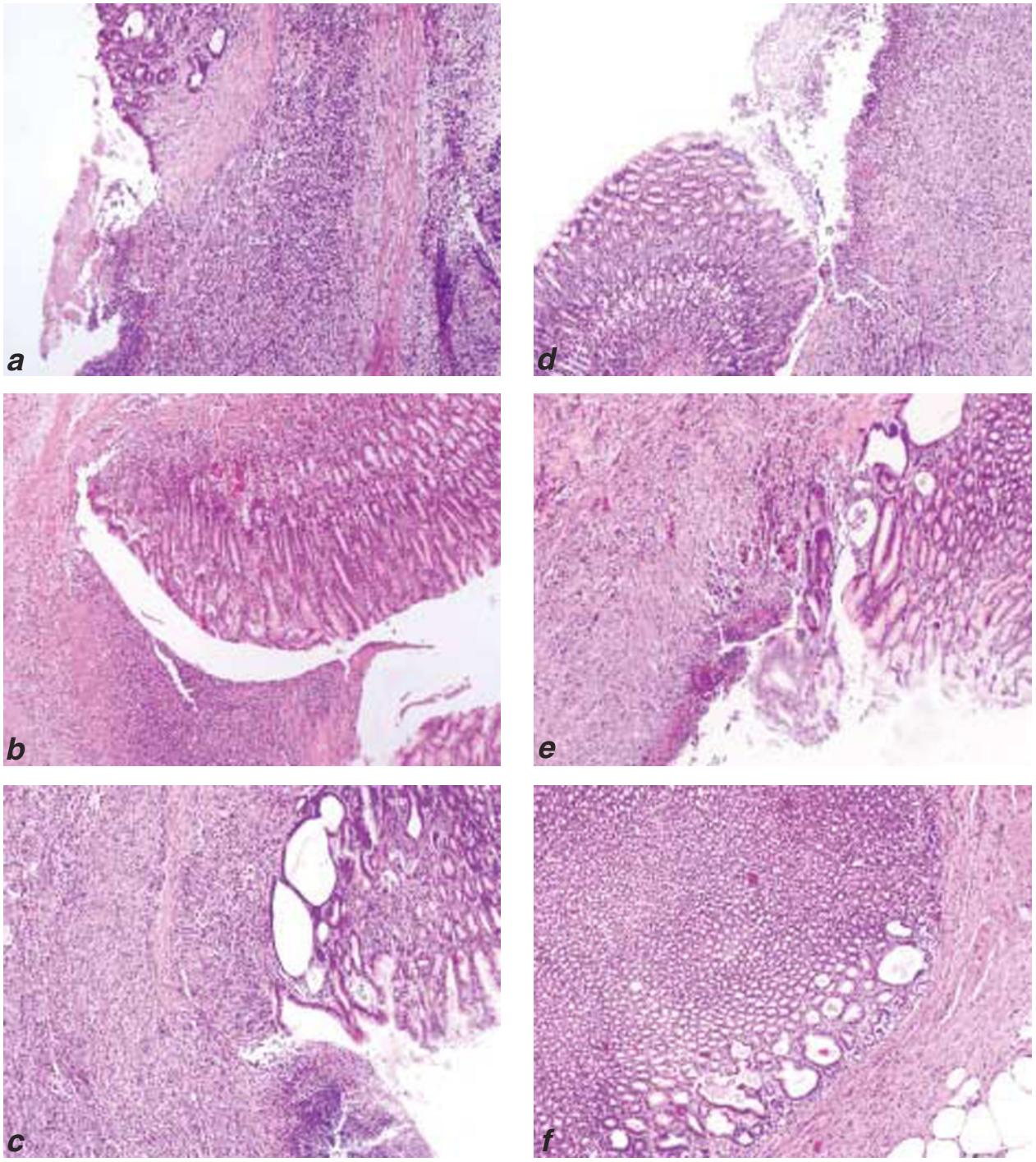

Fig. 3. Histological picture of gastric ulcers in the dynamics of reparative process. *a-c*) on days 10, 20, and 30 after injection of physiological saline, respectively; *d-f*) on days 10, 20, and 30 after transplantation of bone marrow MMSC, respectively; Hematoxylin and eosin staining, ×400.

studies (Fig. 3). In the control group, a thick leukocyte-necrotic layer was found on the bottom of the ulcer on day 10. Under this layer, granulation tissue with abundant thin-walled vessels and predominance of fibroblasts was seen. In deeper layers, this granulation tissue contained partially ordered collagen fibers and small number of blood vessels. In the granulation tissue adjacent to the necrotic zone and its deeper layers, diffuse inflammatory infiltration with predominance of neutrophils was noted (Fig. 3, *a*). In animals receiving transplantation of bone marrow MMSC, this leukocytic-necrotic layer was thinner. The adjacent layer was presented by maturing granulation tissue with low number of

vessels and weak or moderate diffuse leukocyte infiltration consisting primarily of histiocytes, lymphocytes, and neutrophils. Dilated cystic glands with proliferating epithelium were seen at the edges of the ulcer defect (Fig. 3, *a*).

On day 20, the size of ulcers slightly decreased, but no qualitative differences from the previous term were found (Fig. 3, *b*). After transplantation of bone marrow MMSC, the edges of the ulcer defect were partially epithelialized. A thin leukocytic-necrotic layer was found on the bottom of ulcer. The number of blood vessels increased compared to the previous term, collagen fibers were more orderly arranged, weak inflammatory infiltration was presented by lymphoid cells and histiocytes (Fig. 3, *b*).

On day 30, ulcer defects were still seen in control animals. The bottom of the ulcer was coated with a thin necrotic layer and diffusely infiltrated with leukocytes. In the deeper layers, the maturing granulation tissue was characterized by orderly arranged collagen fibers and moderate inflammatory infiltration with lymphocytes, histiocytes, and neutrophils. Dilated cystic glands lined with proliferating epithelium were seen at the edges of the ulcer (Fig. 3, *c*). After transplantation of bone marrow MMSC, epithelialization of the ulcer defect was observed. No ulcer defects were seen in the mucosa, but superficial erosions were observed in some animals; zones with glandular cavities lined with proliferating epithelium were found. Fibrous tissue with ordered collagen fibers and weak inflammatory lymphocyte-histiocyte infiltration was seen in the stromal layer of the mucosa (Fig. 3, *c*).

Histological study revealed a correlation between more rapid and complete regeneration of the ulcer defects and improved microcirculation in animals receiving transplantation of bone marrow MMSC. For instance, 0.4-0.5-mm^2 ulcer defects were still seen in control animals 6 months after NHGU modeling, while in animals receiving transplantation of bone marrow MMSC no ulcer defects and cicatrical changes in the mucosa were found, the mucosa looked rose and evenly epithelialized. We concluded that high quality of regeneration of the ulcer defect was determined by more complete recovery of the structure and regulation of the vascular bed not only in the zone of the ulcer defect, but also in adjacent tissues.

Thus, the following conclusions can be made. NHGU is characterized by inhibition of neoangiogenesis, impairment of microcirculation in the zone of the ulcer defect, and inhibition of reparative regeneration. Bone marrow MMSC delivered to the zone of the ulcer defect stimulate neoangiogenesis, correct microcirculatory disturbances in the gastric mucosa, and promote ulcer healing. Stimulation of neoangiogenesis and improvement of microcirculation under the effect of bone marrow MMSC promote better recovery of the circulatory bed and formation of a full-value regenerate.

The authors are grateful to Prof. O. V. Makarova (Laboratory of Immunomorphology of Inflammation, Institute of Human Morphology, Russian Academy of Medical Sciences) for her help in morphological analysis of the results.

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