Effects of Autologous Bone Marrow Cells on Apoptosis and Regeneration of Non-Healing Autoimmune Gastric Ulcers

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The effects of cultured autologous bone marrow multipotent mesenchymal stromal cells on apoptosis and healing processes were studied on the model of non-healing gastric ulcers in rats. Bone marrow multipotent mesenchymal stromal cells inhibited apoptosis in epitheliocytes of the gastric mucosa and stimulated ulcer healing.

Key Words: *bone marrow cells; chronic gastric ulcer; ulcer regeneration*

High apoptotic activity of the epithelium and imbalance of epitheliocyte proliferation and differentiation promote disorders in reparative regeneration and chronic degeneration of non-healing gastric ulcers (NHGU) [8,9]. Immune dysregulation and cytokine imbalance (increased levels of proinflammatory cytokines) associated with the development of NHGU act as inductors of apoptosis of epitheliocytes and lymphocytes (mainly T cells), which results in chronic course of the ulcerative process [5-7,10].

Based on these assumptions, we attempted using bone marrow cells for regulation of epitheliocyte apoptosis and for induction of healing of autoimmune NHGU, because the bone marrow is the major organ of immunogenesis and its cells are characterized by not only immunocorrective and morphogenetic, but also by angiogenetic and antiapoptotic effects [12,13].

Functional and bioregulatory activities of bone marrow (BM) stem and progenitor cells decrease in chronic diseases (chronic pathological stress) [1], but preliminary *in vitro* culturing in nutrient media promotes recovery of their activity.

We studied the possibility of using cultured autologous BM multipotent mesenchymal stromal cells (MMSC) for regulation of epitheliocyte apoptosis and regeneration of autoimmune NHGU.

MATERIALS AND METHODS

Experiments were carried out on 30 male Wistar rats (250-300 g) in whom autoimmune NHGU [1] without tendency to healing for 2.5 months and longer was modeled. All experimental animals were divided into 2 groups: *1*) experimental group (n=15) included animals with chronic NHGU receiving autologous MMSC; and *2*) control group (n=15) comprised rats with chronic NHGU receiving injection of saline. Bone marrow cells and MMSC monolayer in the course of culturing were obtained by the standard method [4].

On day 45 after induction of NHGU, laparotomy and gastrotomy were carried out; experimental animals were transplanted precultured BM MMSC (suspension of 4×10^6 cells in 0.5 ml saline) by 4 injections around the ulcer in sites located at the same distance from the defect. Controls received similar injections of 0.5 ml 0.9% NaCl.

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The efficiency of NHGU healing after MMSC transplantation was evaluated by the planimetric and morphological methods on days 10, 20, and 30 after transplantation. Paraffin sections (5 μ) for morphological studies were stained with hematoxylin and eosin.

In order to confirm the relationship between NHGU regeneration rate and activity of epitheliocyte apoptosis in the gastric mucosa, apoptosis index (AI) was estimated as the proportion of apoptosis marker (caspase-9) expression in gastric mucosal epitheliocytes per 100 epitheliocytes in a visual field for 5 randomly selected visual fields at ×400. Caspase-9 was selected as a key enzyme initiating apoptosis and a transmitter of the mitochondrial pathway of apoptotic signaling [3,11].

AI was estimated in 3 compartments of the gastric mucosa: in the surface pit epithelium; in the pericervical zone (isthmus, proliferative compartment); and in deep zone of the gastric glands (middle and lower thirds of the glands). Sections (3 μ) were prepared and immunohistochemical studies were carried out by the streptavidin-biotin method (Novostain Universal Detection Kit; Novocastra). Rat monoclonal antibodies to caspase-9 (Abcam, Cat. No. ab.32539) in standard dilution 1:70 were used. The number of epitheliocytes expressing the apoptosis marker (brown staining) was evaluated under an Axiovert 100 microscope (Zeiss).

In order to verify the relationship between activities of the regeneration and apoptosis processes and immune balance in the body, we measured serum concentrations of cytokines (TNF-α, IL-1β, IFN-γ, IL-4, IL-10, and transforming growth factor-β; TGF-β) by enzyme immunoassay using Diamed test system before inducing NHGU and over the course of treatment with MMSC.

The results were processed using Student's *t* test.

RESULTS

On day 10 after MMSC transplantation, the area of ulcerative defect shrank to 250±37 mm^2 in comparison with the control, in which the ulcers did not change in size (450±51 mm^2).

The difference in the size of ulcers became significant on days 20 and 30 after MMSC transplantation. By day 30, the ulcers completely healed in the experimental group, while in the control group the ulcers remained large (130±21 mm^2).

Histological picture of NHGU also changed. In the control group, the leukocytic necrotic layer on the bottom of the ulcer on day 10 was more extensive. Granulation tissue with few fine-walled blood vessels and predominating fibroblasts was detected in the sublayer. In deeper compartments, the granulation tissue was characterized by ordered arrangement of collagen fibrils and the presence of few vessels. Pronounced diffuse inflammatory infiltration with predominating neutrophils was seen in the layer adjacent to the necrotic zone and in deeper layers of the granulation tissue.

In experimental animals, the destructive inflammatory phase of the wound process was sooner (on day 10) replaced by the proliferative regeneratory phase. This was seen from the presence of thin leukocytic necrotic layer, appearance of the granulation tissue with numerous vessels, and moderately diffuse leukocytic infiltration (predominantly histiocytes and lymphocytes, and also neutrophils). Cyst-like dilated glands with proliferating epithelium were detected in the marginal zone of the ulcerative defect.

In the controls, the ulcers somewhat shrank by day 20, but there were no pronounced histological changes in comparison with the previous period. Histological studies of ulcers in animals receiving MMSC showed partial epithelialization of ulcer ed-

TABLE 1. Dynamics of Epitheliocyte Apoptosis Index in the Gastric Mucosa of Animals with NHGU after MMSC Transplantation and without It (*m*±*m*)

Group	Day of study	Surface pit epithelium	Pericervical zone (isthmus, proliferative compartment)	Gland base (middle and lower thirds of glands till basal compartments)
Control, 0.9% NaCl	10	34.30±2.82	21.60±0.34	43.20±3.26
	20	28.70±1.74	17.00±1.56	27.90±1.64
	30	17.40±1.16	10.40±1.39	18.80±1.33
MMSC transplantation	10	21.90±2.48	12.30±2.11*	32.30±2.73
	20	11.10±0.82*	7.90±0.45*	14.50±1.79*
	30	5.50±0.25*	2.19±0.23*	6.70±0.38*

Note. *$p<0.05$ compared to the control.

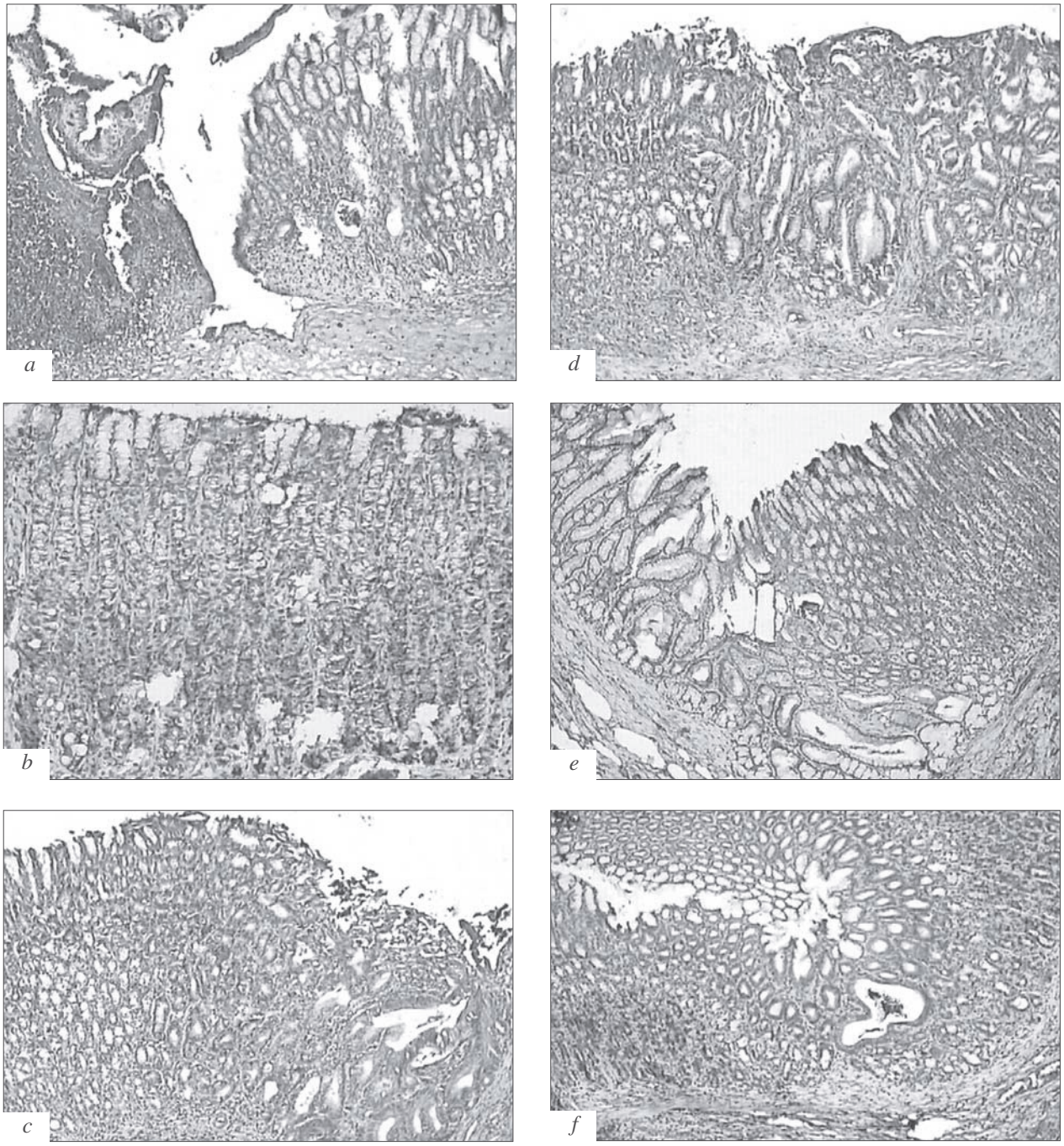

Fig. 1. Expression of caspase-9 in gastric mucosal epitheliocytes. *a-c*) days 10, 20, 30 after injection of saline (control); *d-f*) days 10, 20, 30 after BM MMSC transplantation. ×120 (*a*, *c-f*) ×250 (*b*).

ges by the same term. Thin leukocytic necrotic layer was detected on the bottom of the ulcer. In comparison with the previous period, the number of blood vessels increased in animals of this group; collagen fibrils were more ordered, inflammatory infiltration was minor (mainly by lymphoid histiocytic cells).

On day 30, the ulcer defects persisted in the controls. The ulcer bottom was covered with a thin necrotic layer and was diffusely infiltrated by leukocytes. The maturing granulation tissue in the sublayer was characterized by ordered arrangement of a part of collagen fibrils; inflammatory infiltration by histiocytes, lymphocytes, and neutrophils was moderate. Cyst-like dilated glands lined with proliferating epithelium were seen in the ulcer edges. In the experimental group, the ulcers virtually healed macroscopically and the defect epithelialized by

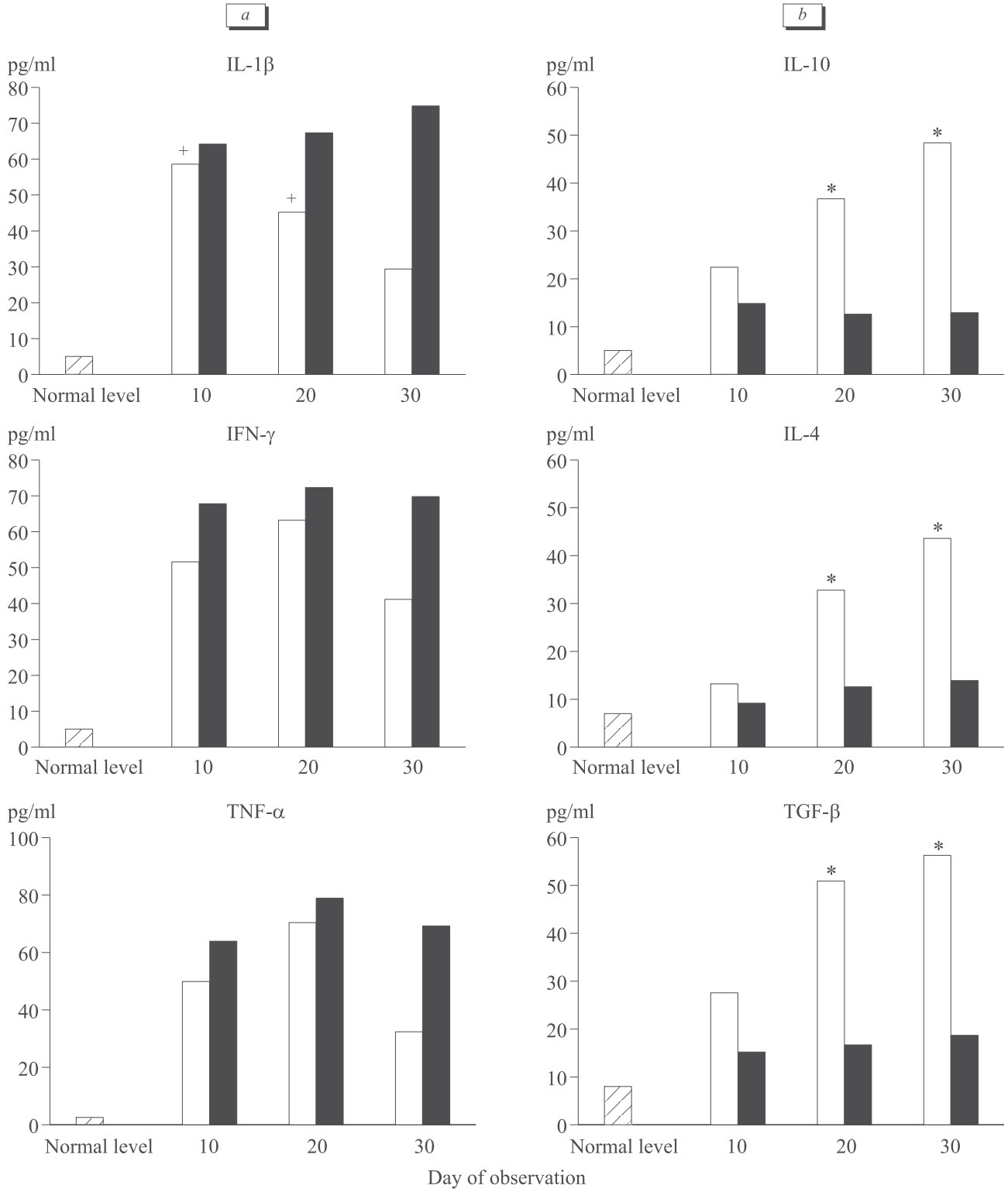

Fig. 2. Changes in the production of proinflammatory (*a*) and antiinflammatory (*b*) cytokines in the sera of rats with autoimmune NHGU after transplantation of BM MMSC into the ulcer zone. Light bars: after BM MMSC transplantation; dark bars: after injection of saline (control). Abscissa: cytokine content (pg/ml); ordinate: day of observation. *$p<0.05$ compared to the control.

day 30 of observation. No ulcerative defects were detected in the mucosa; there were just zones with glandular cavities lined with proliferating epithelium. Fibrous tissue with orderly arranged collagen fibrils and mild inflammatory lymphoid histiocytic infiltration was detected in the underlying stroma.

Acceleration of ulcer regeneration under the effect of MMSC was due to regulation of apoptosis activity in the gastric mucosa, which was seen from reduction of AI (level of apoptosis marker expression) in three compartments of the gastric mucosa during treatment (Table 1; Fig. 1). On the other hand, AI remained high in NHGU without MMSC transplantation. In controls, a pronounced expression of apoptosis marker in epitheliocyte cytoplasm of all compartments of the gastric mucosa was observed on day 10 (Fig. 1, *a*), and hence, AI was high (Table 1). In the experimental group, expression of the apoptosis marker was less pronounced (Fig. 1, *d*) and AI tended to decrease during the same period. Evaluation of the expression of apoptosis marker in individual compartments in both groups showed significant differences (Table 1). A significantly reduction of AI on day 10 after BM MMSC transplantation was noted in epitheliocytes of the pericervical zone of the gastric mucosa. In controls, AI remained high in this zone during this period. The expression of the apoptosis marker was significant decreased and AI of epitheliocytes in three compartments was reduced on days 20 (Fig. 1, *e*) and 30 (Fig. 1, *f*) after BM MMSC transplantation, which seemed to indicate normalization of cell regeneration without inflammatory infiltration during activation of differentiation processes at the final stage of the regeneration. In controls, the expression of the apoptosis marker and AI of epitheliocytes decreased slower and remained high on days 20 and 30. This indicated persisting chronic inflammation in the gastric mucosa of controls and a sluggish course of regeneration.

Positive dynamics of the regeneratory process and reduction of apoptosis activity in the gastric mucosa of animals receiving BM MMSC transplantation were attributed to the presence and continuing functioning of MMSC in the ulcer defect zone and to recovery of the cytokine status in these animals (Fig. 2).

The mechanisms of apoptosis are disordered in autoimmune inflammation, which disturbs elimination of autoreactive cell clones. Apoptosis dysregulation is paralleled by a sharp increase in the number of activated lymphocytes directly in the focus of autoimmune disease. We hypothesized that BM MMSC created conditions for regulation of epitheliocyte and lymphocyte apoptosis and reduced autoimmune inflammation by modifying the profile of produced cytokines and by participating in the regulation of differentiation of some of T-cell clones. This presumably led to accumulation of $CD4^+CD25^+$ (T-regulators) and to suppression of proliferation and differentiation of effector cells (cytotoxic T cells, natural killers) and of dendritic cells. This eventuated in inhibition of autoimmune inflammation and activation of reparative regeneration of NHGU.

Elimination of the cytokine imbalance in animals after BM MMSC transplantation creates conditions for reduction of autoimmune inflammation and epitheliocyte and lymphocyte apoptosis, switching over the latter cells to NHGU regeneration.

Hence, the cytokine imbalance in the body and activation of epitheliocyte apoptosis in the gastric mucosa associated with it, are factors inhibiting NHGU regeneration and promoting its chronic degeneration. Autologous MMSC, removing the cytokine imbalance, promote inhibition of epitheliocyte apoptosis in the gastric mucosa, prevent further destructive ulcerative involvement of the mucosa, and promote rapid effective healing of the ulcer defect. Increase in the levels of proinflammatory cytokines (IL-10, IL-4, TGF-β) after MMSC transplantation correlates with reduced expression of apoptosis marker (caspase-9), which indicates recovery of homeostasis in the gastric mucosa.

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