Thrombin Receptor Agonist Peptide Immobilized in Microspheres Stimulates Reparative Processes in Rats with Gastric Ulcer

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The effect of synthetic thrombin receptor (PAR1) agonist peptide encapsulated in microspheres made of lactic and glycolic acid copolymer on tissue reparation was studied in rats with acetate-induced ulcer. PAR1 agonist peptide was immobilized in biodegraded lactic and glycolic acid microspheres by double emulgation, the kinetics of peptide release was analyzed, and the dynamics of ulcer healing was studied in experimental (administration of microspheres with the peptide into the stomach) and two control groups (administration of saline or spheres without peptide). Thrombin receptor agonist peptide gradually released from lactic and glycolic acid microspheres into the stomach shortened the inflammation phase and shifted the proliferation phase to the earlier period, thus accelerating healing of experimental ulcers in rats.

Key Words: *proteinase-activated receptors; thrombin; agonist peptides; inflammation; gastric ulcer*

Thrombin (the trypsin family serine proteinase), the main enzyme in the blood clotting cascade, is formed in sites of vascular injury, incorporates in fibrin clot for protection from inactivation by inhibitors, and is gradually released into the site of injury. Thrombin regulates all stages of tissue reparation: inflammation, cell proliferation, and maturation of tissues [2,6], directly modulates cell migration, proliferation, and production of growth factors and inflammation mediators, realizing its effect through activation of PAR1 thrombin receptor (a subtype of a new family of proteinase-activated receptors PAR) [3]. The mechanism of PAR activation consists in cleavage of N-terminal fragment of the extracellular domain of the receptor molecule under the effect of proteinases and exposure of a new structure of the so-called "tied" ligand. The interaction between the "tied" ligand and PAR triggers activation of effector cells. Synthetic peptides homologous by their structure to the "tied" ligand act as PAR agonists.

The use of thrombin as growth factor in wound healing is not perspective because of mobility of the enzyme, probability of viral contamination, and antiinflammatory effect of high concentrations of thrombin [2]. The use of thrombin receptor agonist peptides simulating the effect of the enzyme on cells seems to be promising. In previous studies PAR1 agonist peptides (PAR1-AP) were immobilized in

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polymeric biodegraded matrices (polymeric composite polyvinylcaprolactam films or polymeric biodegraded microspheres) for preserving the peptide activity and ensuring long-term and controllable release of the peptides into the wound; these preparations were shown to accelerate healing of experimental skin wounds in mice [1,9]. Experiments on rat model of ethanol ulcer showed that PAR1-AP injected intravenously with amastatin (aminopeptidase inhibitor) protected the gastric mucosa in mild injuries [4]. Moreover, the protective effect of PAR2 agonist peptide (2-furoyl-LIGRL) was demonstrated on the model of gastric mucosa injury induced by ethanol and HCl [5].

We studied the effect of PAR1-AP immobilized in biodegraded lactic and glycolic acid copolymer capsules on healing of acetate-induced gastric ulcer in rats.

MATERIALS AND METHODS

Synthetic PAR1 agonist peptide SFLLRN (PAR1-AP, Cardiology Research Center, Ministry of Health of the Russian Federation, Moscow) encapsulated in microspheres based on (d,1)-lactic and glycolic acid copolymer by double emulgation [8] was used in the study. The first emulsion was prepared by mixing gelatin solution (G2500, type III, Sigma) with PAR-AP solution; the resultant aqueous phase after homogenization was dispersed into hydrophobic phase, PLGA(P(d,1)LA-GA solution (50:50; RG 503, mol. weight 29,000; Boehringer Ingelheim) in methylene chloride (Sigma) at 40°C. Then the resultant first emulsion was homogenized, cooled to 15°C, and added (with constant stirring) to aqueous solution of polyvinyl alcohol (3-83, mol. weight 18 000, Hoechst). After 15-min incubation of microspheres in polyvinyl alcohol solution the temperature was elevated to 30°C and the solvent (methylene chloride) was evaporated. The resultant microspheres were washed on a filter and lyophilized. Destruction of microspheres with immobilized PAR1-AP was studied by light and electron microscopy during their incubation in phosphate buffered saline at 37°C and in solution containing 0.1 M HCl and 0.5% sodium alginate solution (Sigma). The mean size of the capsules was 30-40 μ. The kinetics of desorption of encapsulated PAR1-AP was evaluated by its capacity to induce human platelet aggregation (*in vitro*) [1].

Experiments on ulcer healing were carried out on 25 outbred male rats using modified Okabe method. All manipulations on animals were carried out in accordance with the Helsinki Declaration on humane handling of animals.

The animals were narcotized with ether and glacial acetic acid was applied onto the gastric serosa (1×1 cm^2). Two hours after surgery the experimental animals (n=9) received (into the stomach, through a tube) 5 mg microspheres with PAR1-AP in 300 μl 0.5% sodium alginate. Group 1 controls received saline (500 μl/200 g body weight; n=6), group 2 controls received capsules without peptide (n=10). Morphometry of the tissue specimens from the site of injury collected on days 3 and 7 of experiment was carried out. Histological sections of the damaged gastric tissue were stained with hematoxylin and eosin. The intensity of reparative process in the submucosa was evaluated by the percentage of macrophages and neutrophils (inflammation markers) and fibroblasts (proliferation marker). The significance of differences was evaluated using Student's t test.

RESULTS

The study of the destruction kinetics of PLGA microspheres with immobilized PAR1-AP in phosphate buffer revealed changes in capsule structure and injury to microparticles after 2 days of incubation; after 7 days degraded particles of microspheres were found. Destruction of PLGA capsules at pH 2.0 was much more rapid: damaged microspheres appeared after 2 h, while after 24 h the capsules were virtually completely destroyed.

The study of the kinetics of PAR1-AP desorption from microparticles showed that 90% peptide was released during the first hours of incubation. Desorbed peptide activated PAR1 receptors on platelets and induced their aggregation, this indicating retained physiological activity of the peptide after desorption.

The capacity of PAR1-AP immobilized in microcapsules to accelerate tissue reparation was studied *in vivo* on a model of gastric ulcer in rats.

In control animals on day 3 after surgery neutrophils (more than 70%) and macrophages (24.9% in control group 1, 23% in control group 2) predominated among cells in the exudative layer of gastric ulcer (Fig. 1). Fibroblasts were found as solitary cells against the background of massive neutrophil and monocyte/macrophage infiltration, edema, unevenly plethoric vessels in both control groups (Fig. 1). No significant differences in the cell composition of the gastric submucosa were detected in these control groups.

In experimental animals the inflammatory reaction and edema were less pronounced on day 3 than in control rats: the number of cells with typical signs of fibroblast differentiation increased significantly, presumably indicating accelerated matura-

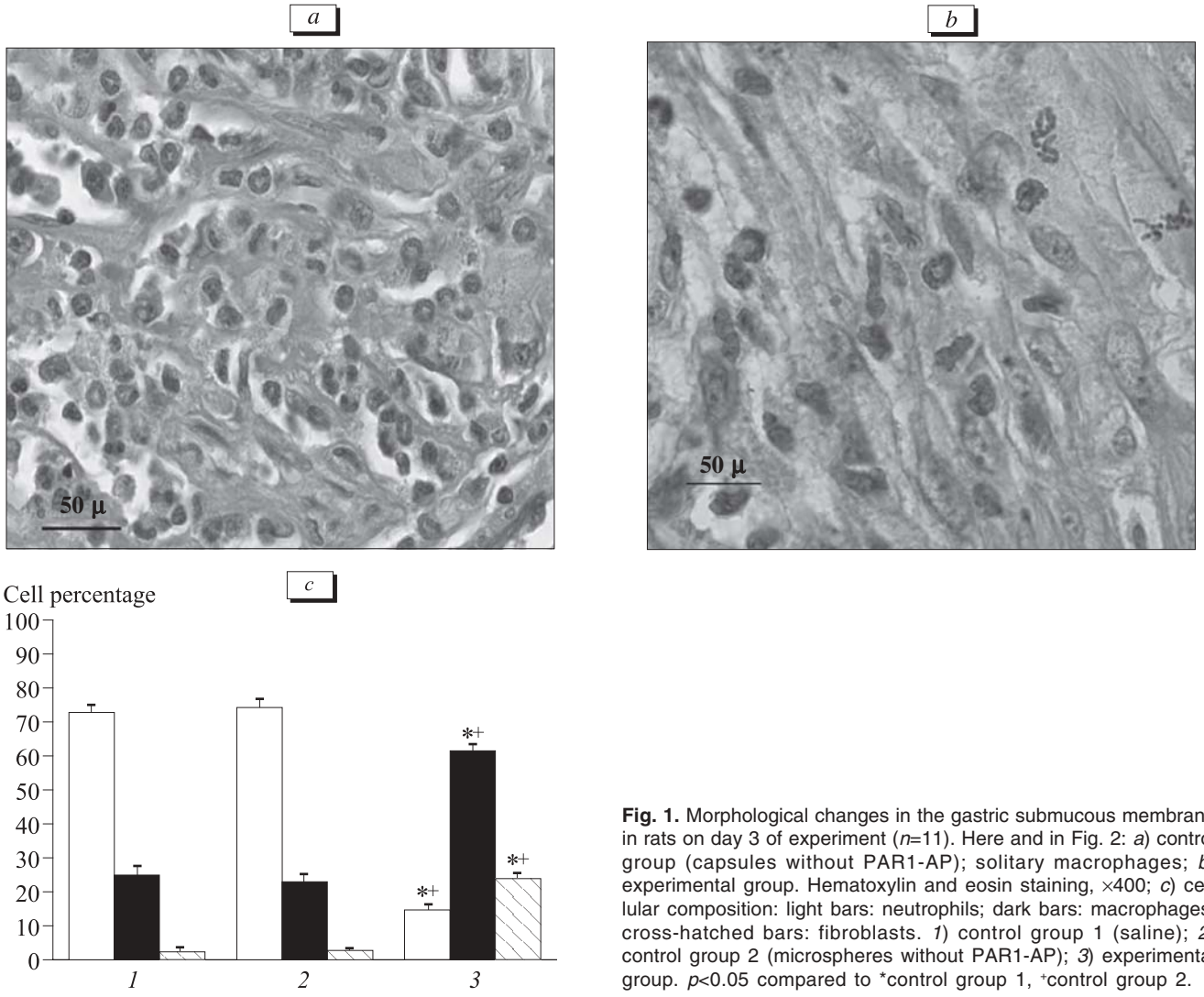

Fig. 1. Morphological changes in the gastric submucous membrane in rats on day 3 of experiment (n=11). Here and in Fig. 2: *a*) control group (capsules without PAR1-AP); solitary macrophages; *b*) experimental group. Hematoxylin and eosin staining, ×400; *c*) cellular composition: light bars: neutrophils; dark bars: macrophages; cross-hatched bars: fibroblasts. *1*) control group 1 (saline); *2*) control group 2 (microspheres without PAR1-AP); *3*) experimental group. p<0.05 compared to *control group 1, +control group 2.

tion of fibroblast precursors migrating into the wound area (Fig. 1). In experimental group the count of neutrophils was 5-6-fold lower, while that of macrophages 3-fold higher than in control groups. The content of fibroblasts was 23.9%, *i.e.* more than 10-fold surpassed the corresponding parameter in the control groups (Fig. 1, *c*). Hence, sooner onset of the healing phase was observed in experimental animals due to concentration and proliferation of fibroblasts in the regeneration zone .

On day 7 after surgery clearly oriented fibroblasts predominated in the granulation tissue of experimental animals, loose network of intercellular matrix appeared around these cells (Fig. 2, *b*). Greater number of formed fine-wall capillaries was seen in the wound zone, in comparison with the controls; a statistically significant decrease in the counts of neutrophils and macrophages and a more than 50% increase in the content of fibroblasts were observed (Fig. 2, *c*).

Hence, our findings suggest that PAR1-AP encapsulated in polymeric microspheres activated migration and proliferation of fibroblasts and endothelial cells in experimental ulcer. PAR1 thrombin receptor agonist peptide regulated cell functions in the focus of injury during the inflammatory and proliferative phases of wound healing, shortened the inflammation phase, and promoted the transition to the proliferation phase, thus accelerating healing of experimental gastric ulcers in rats.

The effects of thrombin and its PAR1 receptor agonist on target cells were direct and mediated through expression of growth factors, stimulating leukocyte and fibroblast migration into the focus of lesion and initiating cell proliferation and differentiation [2,7]. Incorporation of PAR1 agonist in polymeric matrices preserved and optimized its activity in the wound due to gradual regulated release. New biocompatible and biodegraded carriers are needed, capable of releasing labile PAR1 agonist peptides

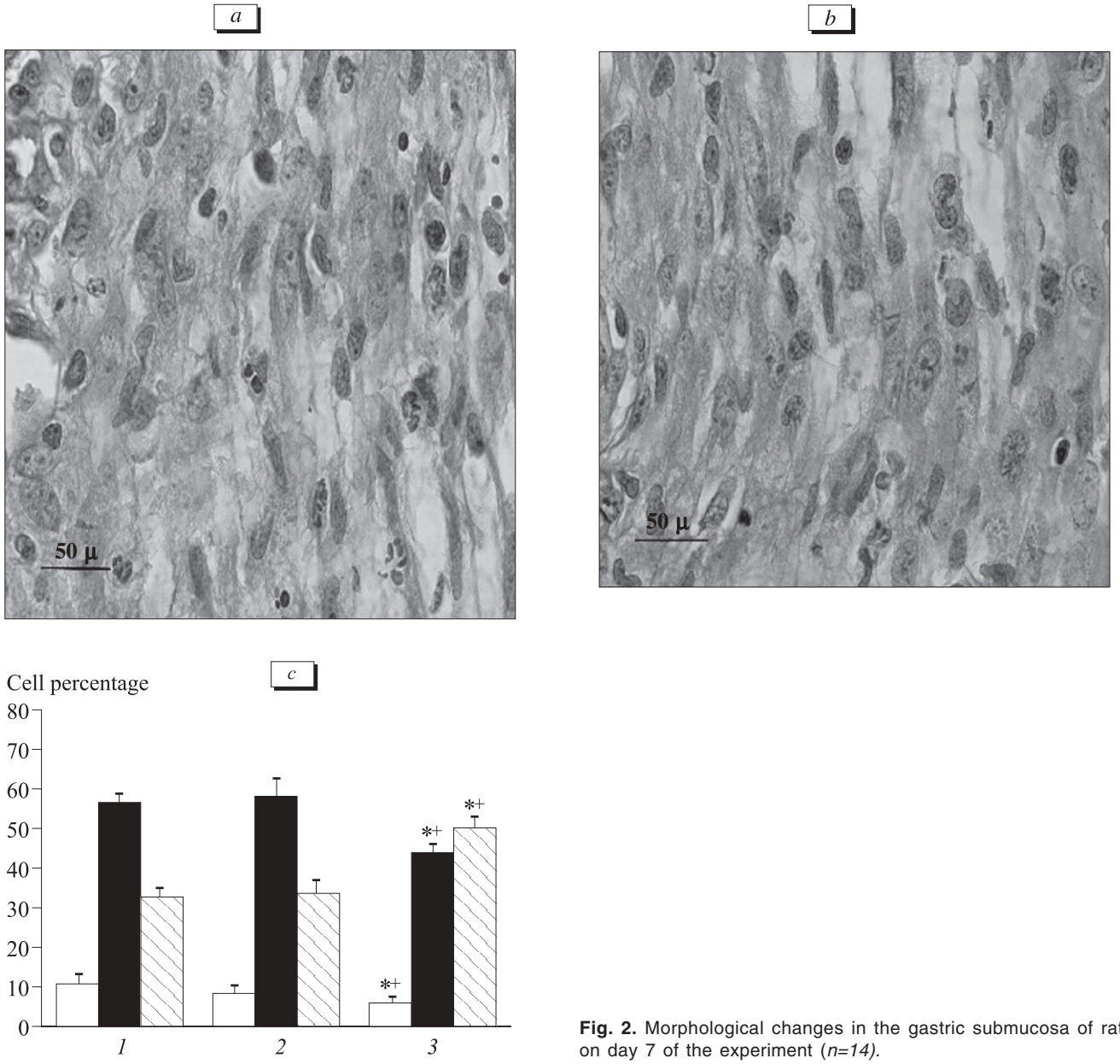

Fig. 2. Morphological changes in the gastric submucosa of rats on day 7 of the experiment (*n=14).*

at the site of tissue injury with preset regulated rate. Biocompatible and biodegraded lactic and glycolic acid copolymer is perspective from this viewpoint.

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