Effect of Synthetic Peptide Thrombin Receptor Agonist Encapsulated in Microparticles Based on Lactic and Glycolic Acid Copolymer on Healing of Experimental Skin Wounds in Mice

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PAR1 peptide thrombin receptor agonist (PAR1-AP) was encapsulated in microcorpuscles based on lactic and glycolic acid copolymer. The desorption profile of the preparation was studied *in vitro* and its wound-healing effects were studied on a model of cut skin wound in mice. The study showed that 90% PAR1-AP was desorbed over 6 h, but the peptide was detected in eluates from the microparticle surface after 23 h. The desorbed peptide retained its physiological activity and was capable of activating PAR1 receptors on human platelets. The study of the dynamics of experimental skin wound healing in mice showed lower number of macrophages in the wounds treated with PAR1-AP microparticles compared to the control (open wounds and wounds covered with microparticles) and higher number of fibroblasts on day 3 of tissue reparation. Hence, PAR1-AP desorbed from microparticles shortened the inflammation phase in the wound. On day 7 the best healing parameters were also observed in wounds treated with PAR1-AP microparticles, which attests to shortening of the proliferation phase and acceleration of wound healing.

Key Words: *protease activated receptors; thrombin; peptide agonists; wound healing*

Expression of protease-activated receptors (PAR family) on different cell types (platelets, endotheliocytes, fibroblasts, smooth-muscle cells, *etc.*) determines the bioregulatory functions of serine proteinases in the inflammation and proliferation processes during tissue reparation [4]. The mechanism of PAR activation consists in proteinase-induced cleavage of the N-terminal fragment of the receptor extracellular domain and exposure of the new structure, the so-called "bound" ligand. The interaction between bound ligand and PAR receptors triggers activation of effector cells [2]. Synthetic peptides with the structure homologous to this ligand act as PAR agonists.

Thrombin (serine proteinase) forms in damaged vessels, binds to fibrin clot (this protects thrombin from inactivation with inhibitors), and is gradually released into the site of injury regulating all stages of tissue reparation [1]. In 1993 D. H. Carney *et al.* [7] for the first time detected thrombin capacity to accelerate wound healing and isolated a fragment of enzyme molecule (TRAP508) inducing nonproteolytic activation of reparation. We previously showed that both

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thrombin and synthetic peptide — its PAR1 receptor agonist, encapsulated in polymeric wound dressing (composite alginate-chytosan polyvinylcaprolactam films) are gradually desorbed and stimulate healing of experimental skin wounds in mice [8]. Prospects of using more safe, cheap, and stable (in comparison with thrombin) PAR1 agonist peptides for wound healing in practical medicine prompted further search for biocompatible and biodegraded carriers capable of releasing labile peptides at preset and controlled rate into the site of tissue damage. The lactic and glycolic acid copolymer is perspective from this viewpoint. It is biocompatible and biodegraded, the rate of the release of encapsulated agents can be regulated by modifying the proportion between lactic and glycolic acids.

We studied the possibility of encapsulating PAR1 agonist synthetic peptide in the microparticles based on the (d,l)-lactic and glycolic acid copolymer, investigated its the desorption profile *in vitro* and the wound healing effects on the model of cut skin wound in mice.

MATERIALS AND METHODS

Synthetic PAR1 agonist peptide SFLLRN (PAR1-AP, Russian Cardiology Research and Production Complex) was encapsulated in microparticles based on (d,l)-lactic and glycolic acid copolymer by the method described previously [7].

The kinetics of desorption of encapsulated PAR1-AP was detected by its capacity to induce human platelet aggregation. Platelet aggregation activity was studied in normal human platelet-rich plasma on a Biola aggregometer and evaluated by the maximum radius of aggregates (R_{max}). Microparticles (10 mg) containing PAR1-AP (7 mM) were put into 200 µl 10 mM HEPES-NaOH buffer solution (pH 7.2) and incubated in a water bath (37°C). The supernatant was collected after 2, 4, 6, and 23 h and its capacity to stimulate platelet aggregation was studied; a fresh portion (200 µl) of buffer solution was added to the precipitate and the incubation was continued. The amount of functionally active desorbed peptide was evaluated by the calibration curve reflecting the relationship between R_{max} and PAR1-AP concentration.

The experiments on wound healing were carried out on 12 female (C57Bl/6×CBA)F_1 mice. Microparticles with the peptide were applied onto the wound (1×1 cm^2) in animals (n=4) under Nembutal narcosis immediately after surgery. In controls the wounds were either covered with microparticles containing no peptide (n=4) or left open (n=4). After surgery the animals were placed in separate boxes. The time course of wound healing was analyzed under a light microscope on days 3 and 7. Specimens of granulation tissue from the wound were collected for histological studies. Analysis of impressions was carried out for express diagnosis of the healing process. The impressions were fixed in methyl alcohol, dried, and stained with Azur-eosin by the method of Giemsa [1]. Sections of granulation tissue were fixed in a mixture of ethanol, formalin, and acetic acid and stained routinely with hematoxylin and eosin. The intensity of reparative process was evaluated by the number of macrophages (inflammation marker) and fibroblasts (proliferation marker). The wound area was evaluated directly after the wound was inflicted and on day 7 of healing.

RESULTS

Microparticles with immobilized PAR1-AP containing polylactic and glycolic acids (50% each) were obtained by double emulsification; the mean size of the particles was 30-40 µ. The kinetics of PAR1-AP desorption was studied based on the peptide capacity to stimulate platelet aggregation *in vitro*. It was found that 90% peptide was desorbed from the microparticles over 4 h, but after 23 h the peptide was still present in eluates (Fig. 1). The desorbed peptide activated PAR1 receptors on human platelets, and we therefore hypothesized that it retained its physiological activity after desorption.

The possibility of using PAR1-AP immobilized in microparticles as a coating for wound healing was studied *in vivo* on a model of cut skin wounds in mice. The process of wound healing requires cooperation of numerous cells and close regulation of the degradation and regeneration processes. It includes 3 phases: inflammation, proliferation, and maturation of the granulation tissue. The inflammation phase (usually lasting for 3-5 days) is characterized by the appearance of numerous macrophages taking part in acute adaptive response. The proliferation phase is characterized

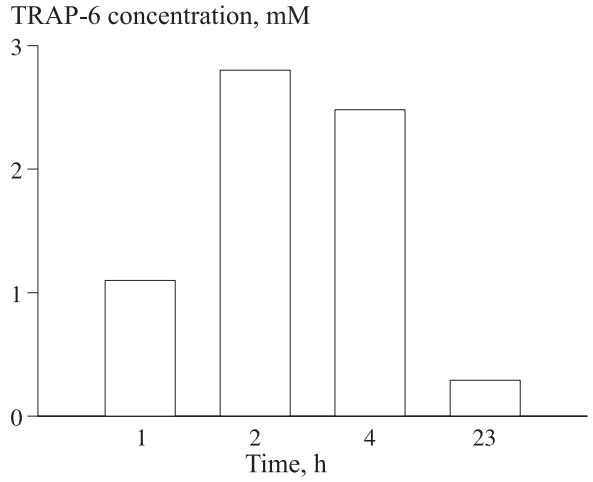

Fig. 1. Dynamics of PAR1-AT release from microparticles.

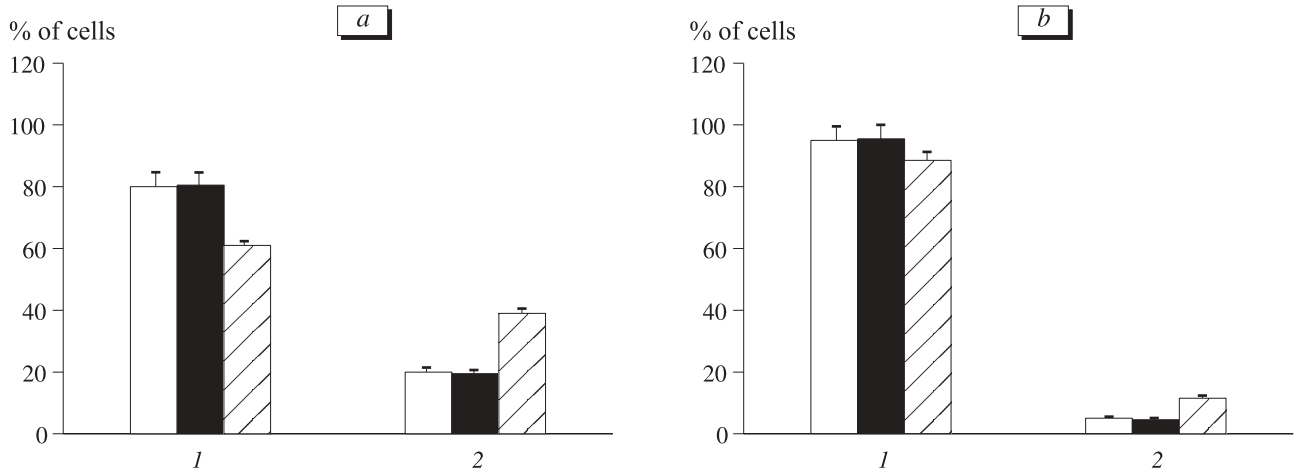

Fig. 2. Effect of encapsulated PAR1-AP on macrophage (*1*) and fibroblast (*2*) content in the granulation tissue of cut wounds in mice on day 3 after wound infliction. Here and in Fig. 3: *a*) sections; *b*) impressions. Light bars: open wound; dark bars: empty microparticles; cross-hatched bars: microparticles with the peptide.

by a sharp increase in the number of proliferating fibroblasts producing collagen and other structural components of extracellular matrix and the formation of vessels in the granulation tissue (angiogenesis). As early as on day 3 of reparation the number of macrophages in the wound covered with microparticles containing PAR1-AP was lower than in the control groups, while the number of fibroblasts was higher (Fig. 2). The sections of the granulation tissue at this term contained 3-4 epithelial layers with numerous mitoses *vs.* 2-3 layers in the control. This attests to shortening of the inflammation phase in the wound under the effect of PAR1-AP desorbed from microparticles. On day 7 after wound infliction the best healing parameters were recorded in the experimental group: maximum fibroblast count and minimum infiltration of the wound with macrophages (Fig. 3). This attests to shortening of the proliferation phase in wounds covered with microparticles containing PAR1-AP and, hence, accelerated healing of these wounds. Seven-eight layers of hornified epithelium were seen on sections of the granulation tissue in these mice, while granulation tissues from open wounds and wounds covered with microparticles had 4 and 5 epithelial layers, respectively.

Impressions of the wound contained free cells not adhering to the matrix, not differing by color, but of different origin (*e. g.* tissue macrophages and blood monocytes). We detected a similar trend in changes of the cell composition seen on impressions and sections during healing (Figs. 2, 3). Impressions can be used for express diagnosis, while analysis of sections can be carried out for a more detailed histological study.

On reparation day 7 the areas of the wounds covered with PAR1-AP microparticles were minimum (8.6, 16.0, and 27.9% of the initial wound area covered with PAR1-AP microparticles, empty microparticles, and open wounds, respectively). Positive wound-healing

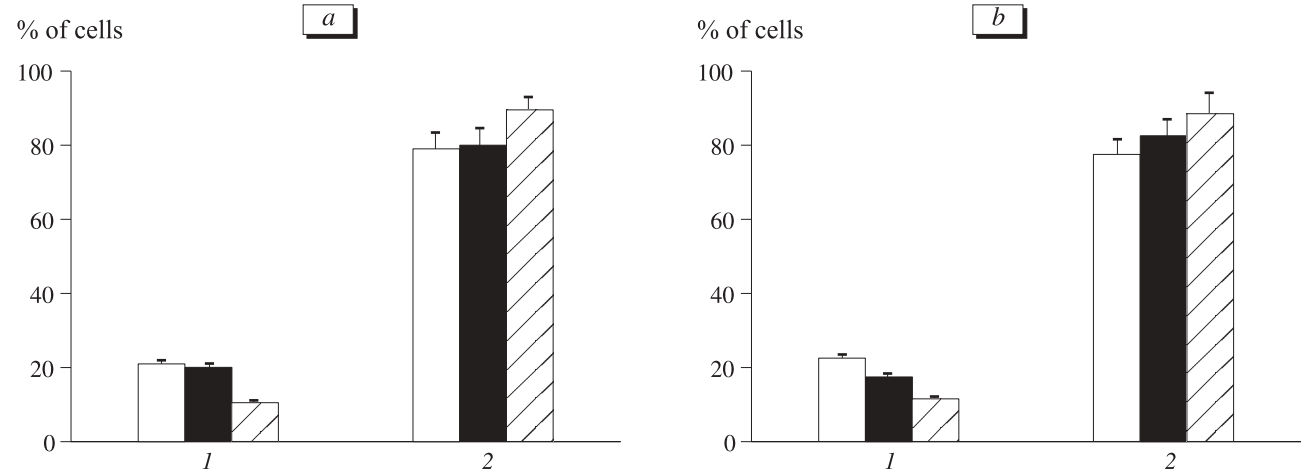

Fig. 3. Effect of encapsulated PAR1-AP on macrophage (*1*) and fibroblasts (*2*) content in the granulation tissue of cut wounds of mice on day 7 after wound infliction.

effect of the lactic and glycolic acid copolymer can be due to protection of the wound from infection.

Hence, exogenous PAR1 thrombin receptor agonist peptide participates in the inflammatory and proliferative phases of wound healing by regulating cell functions in the focus of injury and accelerating wound healing. We found that synthetic PAR1 thrombin receptor agonist peptide encapsulated in polymeric composite polyvinylcaprolactam films accelerated healing of experimental skin wounds in mice [8]. Presumably, thrombin and its PAR1 receptor agonist exert direct and indirect effects (mediated through expression of growth factors causing leukocyte and fibroblast migration into the wound and initiating cell proliferation and differentiation) [1,6]. Encapsulation of PAR1 agonist in polymeric matrices preserves and optimizes its activity in the wound by its gradual controlled release.

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