EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Specific Effect of Epidermal Growth Factor Immobilized on Wound Coating with Soluble Collagen on Healing of Experimental Wounds

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Electron-microscopy and autoradiography of granulation tissue in wounds showed that bioactive coating with epidermal growth factor activates functional and proliferative processes in vascular cells (endotheliocytes and pericytes) and fibroblasts and increases the number of cell-to-cell contacts on days 2-3 of therapy. Pronounced activation of proliferative processes resulted in early differentiation of fibroblast cells (on days 3-4) and rapid epithelialization of the wound.

Key Words: *epidermal growth factor; collagen-based wound coating; regeneration; granulation tissue; electron microscopic radioautography*

None of the known methods of wound healing satisfies the surgeons completely. Special interest to this problem are explained by new data altering the concepts of wound process. Growth factors play the key role in regulation of cell migration, proliferation, and differentiation and deposition of extracellular matrix components during wound healing [2]. The use of recombinant technologies in the production of growth factors opens new vistas in the correction of healing process. Epidermal growth factor (EGF) is a potent stimulator of regeneration, DNA replication, and proliferation of various types of cells [6-8]. However structural and functional changes in granulation tissue locally treated by bioactive dressing with EGF have never been investigated.

Our purpose was a complex study (light and electron microscopy, autoradiography, and morphometry) of wound granulation tissue in experimental animals with aseptic inflammation.

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MATERIALS AND METHODS

Experiments were performed on 40 male Wistar rats. After depilation and iodine treatment, skin fragments no more than 3 cm in diameter were dissected to the underlying fascia and aseptic dressing was applied. After 3 days the crusts were removed and treatment with wound coatings was started. Before dressing the wounds were washed with nitrofurazone (1:5000) and dried with sterile gauze. In the controls wounds healed under collagen films without EGF and in experimental rats under the same film with immobilized EGF. Granulation tissue was examined on days 2, 3, 4, and 7 of treatment, which corresponded to days 5, 6, 7, and 10 of wound healing. For autoradiography, 1-mm^3 fragments were cut from biopsy specimens collected during dressing and incubated at 37°C for 1.5 h in medium 199 with 100 μCi/ml ^{3}H-uridine (specific radio-

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activity 26 Ci/mM) or 20 μCi/ml ^{3}H-thymidine (21.6 Ci/mM). After incubation the material was washed in cold medium 199 and phosphate buffer (pH 7.4), fixed in 2.5% glutaraldehyde and 1% OsO_4, dehydrated in alcohols, impregnated in propylene and epon-araldite mixture, and embedded in epon-araldite. Semithin sections were examined under a light microscope and sites for preparing ultrathin sections were selected. Autoradiographs for electron microscopy were made as described previously [3], stained with uranyl acetate and lead citrate [9], and examined under a Philips CM 10 electron microscope.

For comparative quantitative analysis, fibroblasts, macrophages, vessels, and number of fibroblasts and vessels labeled with ^{3}H-uridine were counted under a light microscope in 30 100-mm^2 visual fields at ×1000.

Arithmetic mean and error were calculated and statistical analysis of variations was performed by qualitative signs. Significance of differences was evaluated using Student's *t* test.

RESULTS

On day 2 of treatment with bioactive coating with EGF (day 5 of wound healing) the demarcation zone consisted of polymophonuclear leukocytes (PMNL), macrophages, bacterial cells, and detritus. High activity of macrophages stimulated the development of fibroblast-rich granulation tissue. The number of macrophages increased negligibly in comparison with their initial count (2.9±0.2 and 2.6±0.2, respectively) and even decreased in comparison with the control (3.3±0.3), but the number of functionally active cells was higher in experimental group, judging from ^{3}H-uridine incorporation in the nuclei.

The granulation tissue on the wound bottom was better developed in experimental group than in the control. It consisted of numerous proliferating fibroblasts and functionally active RNA-producing fibroblasts.

In the controls, granulation tissue was loosened and characterized by lower proliferative and functional activity compared to that in experimental animals.

In experimental group, numerous capillaries, many of which were labeled with ^{3}H-uridine, were seen in the upper layers of the granulation tissue. The total number of vessels and the number of proliferating vessels in experimental group 2.3 and 3-fold ($p<0.05$) surpassed the control values (Fig. 1, *a, b*). The numbers of fibroblasts differed significantly: the total number and the number of proliferating cells in the experimental group 1.5- and 2-fold ($p<0.05$) surpassed the control (Fig. 2, *a, b*). Hence, partial lysis of exogenous collagen resulted in local prolonged release of EGF acting as a mitogen, *i. e.* factor inducing cell proliferation. Collagen degradation products also stimulated activity of cell elements and were utilized as plastic material for newly formed connective tissue [1].

During wound treatment with collagen films containing EGF the number of cell-to-cell contacts, including contacts between functionally active macrophages and fibroblasts, increased in comparison with the control, which played an important role in stimulation and autoregulation of the connective tissue growth [5]. The greatest number of cell-to-cell contacts was observed on days 2 and 3 of treatment, *i. e.* prior to active fibrinolysis (Fig. 3, *a*).

In both groups, cells actively producing RNA and DNA, *i. e.*, characterized by a high level of metabolism and proliferative activity, were located mainly in the capillary wall or close to it (Fig. 3, *b*) and were never seen far from vessels. In experimental group, this structure of granulation tissue corresponded to day 2, while in the control to day 3 of treatment.

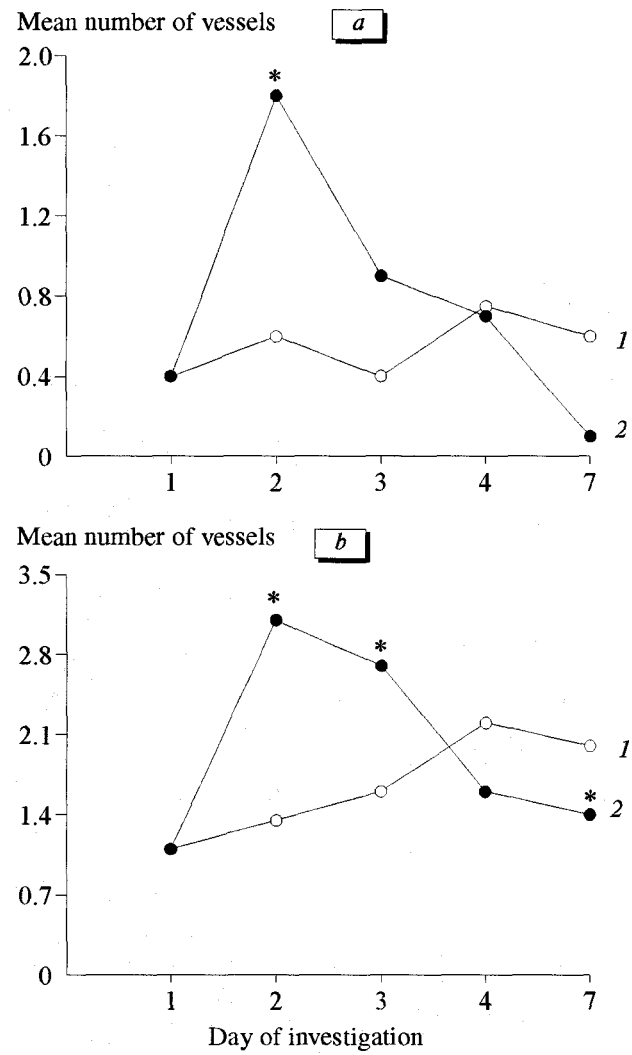

Fig. 1. Effect of bioactive coating with epidermal growth factor on the number of proliferating vessels (*a*) and total number of vessels (*b*) in wound granulation tissue. Here and in Fig. 2: *1*) control; *2*) experiment. *$p<0.05$ *vs.* the control.

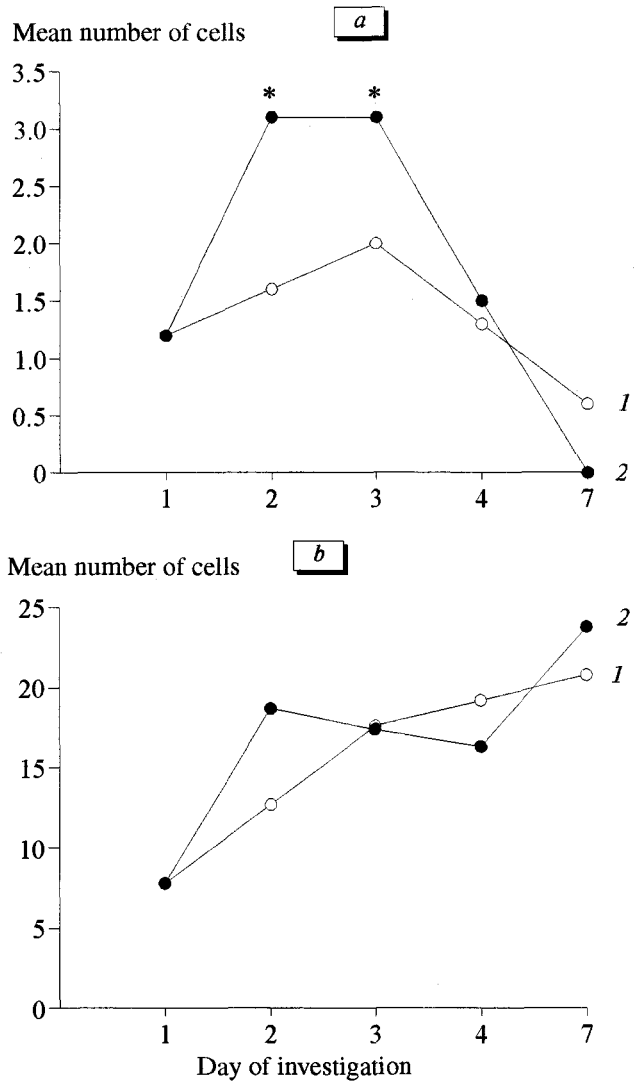

Fig. 2. Effects of bioactive coating with epidermal growth factor on the number of proliferating fibroblasts (*a*) and total number of fibroblasts (*b*) in wound granulation tissue.

Hence, the vessel with adjacent cells is a structural and functional unit of developing tissue with proliferating and differentiating cells.

On day 3 of treatment we observed early differentiation of fibroblast cells due to pronounced activation of proliferative processes. In the experimental group, differentiated fibroblasts (types I and II collagenoblasts) constituted 82.2±3.1% of the total number of fibroblasts ($p<0.05$). Different development of rough cytoplasmatic reticulum and Golgi complex in fibroblasts indicated predominance of different cell functions (accumulation or release of synthesized protein into the extracellular space). In the control, 88.6±3.2% cells ($p<0.05$) were type I collagenoblasts with moderate collagen-producing secretion; no type II collagenoblasts were detected.

The formation of fine collagen filaments and mature fibrils near fibroblasts was observed in both groups.

In experimental group, the number of proliferating capillaries and the total number of vessels gradually decreased (Fig. 1, *a*). The growth of capillaries was paralleled by fibroblast proliferation, which is explained by their histogenetic relationships [4]. The number of microvessels attained maximum on day 2 of treatment, after which they were reduced.

In the control, the total number of microvessels gradually decreased until day 4 of treatment. The processes of vessel growth and fibroblast proliferation were not synchronous (Fig. 1, *b*).

On day 4 of treatment (day 7 of wound healing), the maturation of granulation tissue was in progress in both groups. In the experimental group, fibroblasts were horizontally oriented, their ultrastructural organization attested to high collagen-producing activity; vessels were shaped as vertical loops. Fibroblasts were surrounded by forming collagen structures of different maturity — from solitary fine slightly striated fibrils to large collagen bundles with characteristic periodicity.

In the control, fibroblasts were not so clearly horizontally orientated. Granulation tissue was infiltrated with functionally active macrophages. The number of vessels still increased. Ultrastructure of most fibroblasts was characteristic of collagen-producing cells, and they were surrounded by fine collagen fibrils.

On day 7 of therapy (day 10 of wound healing), wound defect was closed with mature cicatricial tissue in 80% experimental rats, and in 20% a fragment in the center of the wound was not epithelialized. On day 10 of treatment, wound epithelialization was completed in all rats.

In the control, wound defect was filled with mature granulation tissue on day 7 of treatment; on day 10, residual wounds were seen in 70% animals. Wound epithelialization in the control group was completed on day 12 of treatment.

Autoradiography of granulation tissue showed direct and/or indirect effect of EGF immobilized on soluble collagen on the functional and proliferative activity of cells.

We determined morphological criteria of the EGF effect on granulation tissue cells, specifically, on fibroblasts. Fibroblasts are the main source of collagen reproduction in the wound and are involved in the formation of connective tissue, therefore our data are important for the development of new bioactive coatings.

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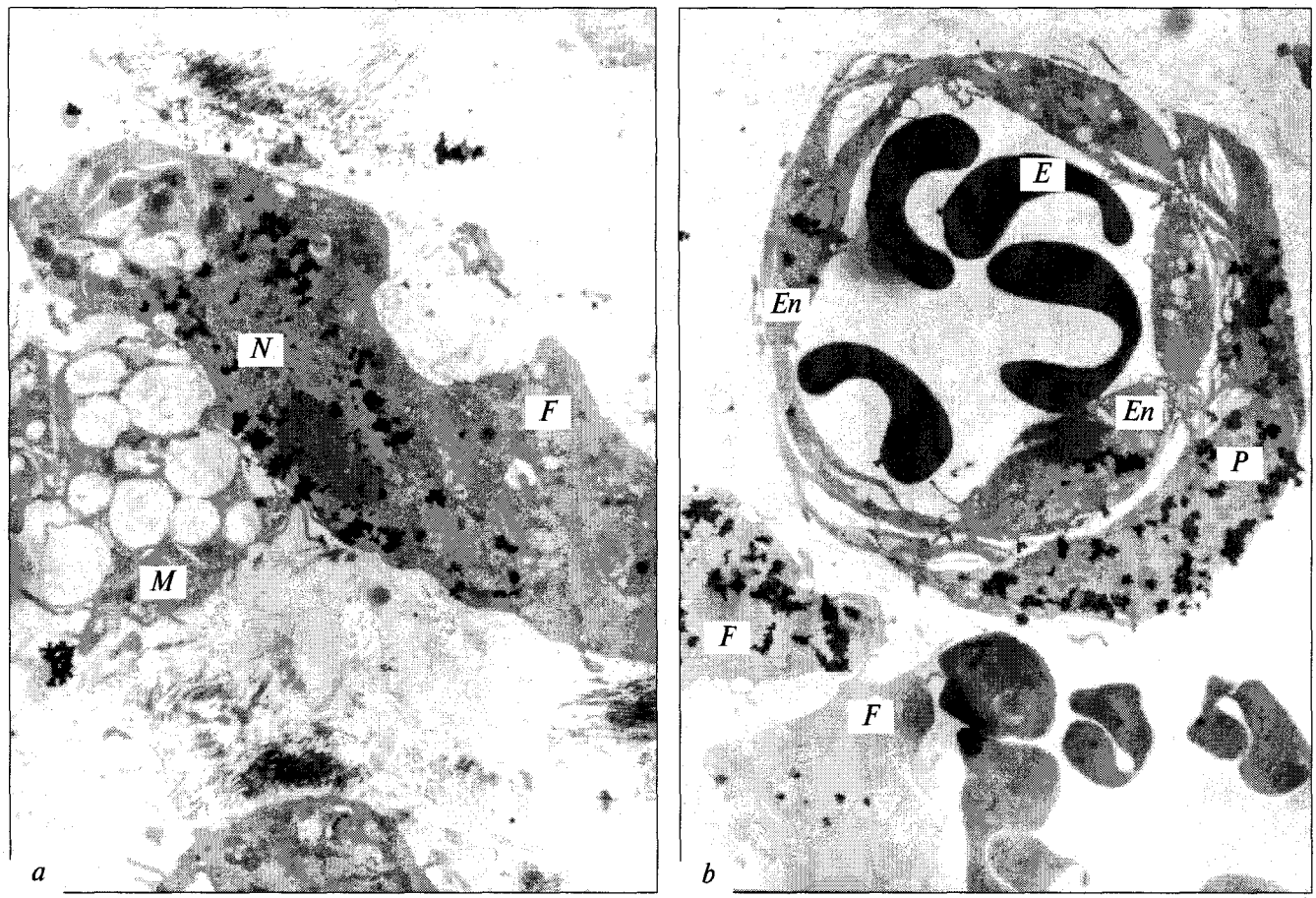

Fig. 3. Granulation tissue from experimental animal on day 2 of treatment with coating containing epidermal growth factor. *a*) cell-to-cell contact between macrophage (M) and fibroblast (F). DNA (black silver grains) production in fibroblast nucleus (N),×8000; *b*) capillary. Erythrocytes (E) in capillary lumen (L). Active DNA production in precapillary endotheliocyte (En) nuclei, pericyte (P), and fibroblasts (F);×12 000.

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