Morphofunctional Evaluation of the Effect of Collagen-1-Based Dressing on Skin Regeneration after Burn Trauma in Mice of Two Genetic Strains

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Morphofunctional evaluation of the effect of biological dressing with collagen-1 on healing of 3A degree burn wound in outbred and mutant Hr^{hr}/Hr^{hr} (hairless) mice was carried out by the histological method and optic radioautography. A pronounced stimulatory effect of the dressing on skin regeneration in mice was demonstrated. According to radioautography data, early dressing of the burn wounds in Hr^{hr}/Hr^{hr} mice led to active proliferation of epithelial cells in dermal cyst and vascular endotheliocytes. The possible mechanisms of the stimulatory effect of collagen-based dressing on wound healing are discussed.

Key Words: *skin regeneration; hairless Hr^{hr}/Hr^{hr} mice; biological dressing; hair follicle; stem cell*

The physiological reaction of the skin epidermis is realized at the expense of its basal layer stem cells. If the basal layer is damaged, stem cells from the skin appendages (hair follicle (HF), sebaceous and sweat glands) are involved in regeneration of the epidermis [12]. Mutation in the *Hairless* (*Hr*) gene in mice causes destruction of HF normally formed during embryogenesis, which leads to complete alopecia in these animals [16]. This mouse strain homozygotic by *Hr* gene mutant allele is used as a model for studies of the mechanisms of HF functioning and for the development of methods for the treatment of hair growth diseases. Phenotypical manifestation of *Hr* gene mutation in mice is similar to human hereditary dermal disease, papular atrichia [17]. Therefore, study of the functional characteristics of *Hr* gene improves our understanding of human skin physiology.

Bioactive dressing materials of different kinds are used for local therapy of burn wounds with good results. Skin wound healing is significantly stimulated by transplantation of living allogenic fibroblasts or application of a collage-1-based dressing with platelet growth factor (PDGF–BB) early (days 1-2) after 3A degree burns in humans [1]. We hypothesized that activation of stem cells (SC) of the skin appendages is one of the mechanisms of the effect of this dressing.

We carried out morphofunctional analysis of the effect of biological dressing with collagen-1 on skin regeneration after 3A degree burns in outbred and mutant hairless Hr^{hr}/Hr^{hr} mice.

MATERIALS AND METHODS

The study was carried out on outbred albino mice (20-25 g) and mutant hairless Hr^{hr}/Hr^{hr} mice (18-22 g). Twenty animals aged 3-5 months were used in the study. Experiments were carried out in accordance with the Order of the Ministry of Health of the USSR No. 755 of August 12, 1975.

Burns (3% body surface) were inflicted to animals intraperitoneally narcotized with avertin (0.25 mg/kg) by application (6 sec) of a 10-mm round signet heated

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to 95-100°C to the skin on the back to a site framed by PVC rings (12 mm in diameter) sutured to the skin in order to prevent wound contraction. According to morphological control, a 3A degree burn was induced. Two symmetrical burns were inflicted simultaneously: one for spontaneous healing and the other for local treatment under biological dressing with collagen-1. After the injury, the animals were kept in individual cages.

Biological dressing (patent of the Russian Federation No. 2314129) consisted of two polymers, natural and artificial. A thin (1 mm) layer of collagen sponge obtained by lyophilization of 1-2% collagen solution was applied onto a sublayer of Carbosil-P organosilicon film perforated for discharge of excessive wound exudation [1]. Collagen-1 was isolated from mouse caudal tendons as described previously [7].

The material for morphological studies was collected 6 h, 1, 3, 5, and 7 days after the injury.

Wound tissue for histological study was fixed in 10% neutral formalin, the sections were stained with hematoxylin and eosin. Functional (protein production) and proliferative activities of wound cells early after the injury (6 and 24 h) were evaluated by radioautography using two low-molecular-weight nucleic acid precursors: ^{3}H-uridine (RNA precursor) and ^{3}H-thymidine (DNA precursor). After plunging in warm nutrient medium 199, the tissue was cut into 0.5-1.0-mm^3 fragments and incubated in nutrient medium with ^{3}H-uridine in a dose of 10 μCi/ml (specific activity 26.0 Ci/mmol) or ^{3}H-thymidine in a dose of 2 μCi/ml (specific activity 21.6 Ci/mmol) at 37-38°C for 1.5 h. The material was fixed in 2.5% glutaraldehyde and 1% osmium tetroxide and embedded in epon/araldite resin.

Radioautographs were made on semithin (1.5-2.0 μ) sections as described previously [3] and stained with toluidine blue. Morphological analysis was carried out under an Axiostar plus light microscope (Zeiss).

RESULTS

Macroscopically, the skin of 3-5-month-old Hr^{hr}/Hr^{hr} mice is characterized by complete absence of hair. Instead of common hair follicles piercing the entire thickness of the skin in outbred albino mice (Fig. 1, *a*) they have structural epithelial formations of two types (Fig. 1, *b*). One type formations are hyperkeratinized sacs bound to the epidermis and often opening into it. Their shape is often abnormal, elongated, "acne-like", and they are bound to the sebaceous gland via a duct. Degrading hair-like filamentous structures can be seen in them. The other type formations are well-developed cysts or bubbles, usually round and enveloped with a monolamellar epithelium located nearby in the derma. However, the deeper they lie, the more irregular indefinite shape they have and the thicker is the epithelium enveloping them, often containing fragments of filamentous structures.

Six hours after 3A degree burns, the mice of both strains had no epidermis and had pronounced destructive changes in the derma with inflammatory reaction (leukocytic infiltration and edema; Fig. 1, *c*, *d*). However, normal mice had HF in the derma, while Hr^{hr}/Hr^{hr} mice had destroyed epithelial sacs and modified dermal cysts. Active proliferation of retained HF epithelial cells was observed in the derma of outbred mice. Similar cellular reaction was observed somewhere in retained epithelium of sacs and dermal cysts of mutant mice.

The reaction of the follicular epithelial cells was more pronounced after application of the biological dressing in mice of both strains. Epithelial cells of HF in outbred mice proliferated, migrated to the wound surface, and lined it (Fig. 1, *e*). In Hr^{hr}/Hr^{hr} mice, the dermal cysts as if regenerated, floated upward, and incorporated in the dermal surface; their epithelial cells also proliferated and migrated, "crawling" onto it (Fig. 1, *f*). Active cell reaction of the follicular epithelium (proliferation and migration) was also observed in the deeper layers of the wound.

Spontaneous healing in outbred mice 1 day after burn was characterized by proliferation of epithelial cell of HF under conditions of dermal destruction and edema; 1 or 2 layers of the epithelium started lining the wound surface (Fig. 2, *a*). There was no epithelium above the sites of burned surface with pronounced destructive changes in the derma (destroyed HF, collagen fibers, and cells; Fig. 2, *c*). Inflammatory reaction presented as focal leukocytic (mainly neutrophilic) infiltration of different degree, often paralleled by bacterial cell phagocytosis. Leukocytes often infiltrated the derma and subcutaneous fat. Separate fibroblasts appeared against the background of a fine network of fibrils 6 h after burn in the depth of the wound at the level of subcutaneous fat. With time, the count of fibroblasts migrating into the derma from the subcutaneous fat increased.

The formation of normal bilayer epithelium was observed on a larger surface of the wounds healing under collagen dressing. Multilamellar (3-4 layers) epithelium was seen in some places, epithelial cells piling one upon the other, this indicating high proliferative activity of the epithelium (Fig. 2, *b*). Although the structural disorders in the derma persisted (destruction of cells and collagen fibrils), no inflammatory events were usually seen in it (Fig. 2, *d*). The derma was a homogeneous layer with capillaries appearing amidst loosely packed collagen fibrils; fibroblasts became predominant cells.

During this period, the epithelium of the sacs and numerous "elevated" cysts in mutant mice (Fig. 2, *t*,

f) was thicker (2-3 layers) and actively developed in the wound surface, sometimes crawling under the crust in the wounds treated under collagen dressing. High proliferative activity of epithelial cells was seen from their chaotic dissemination and piling onto each other in HF derivatives and in the new "crawling" epithe-

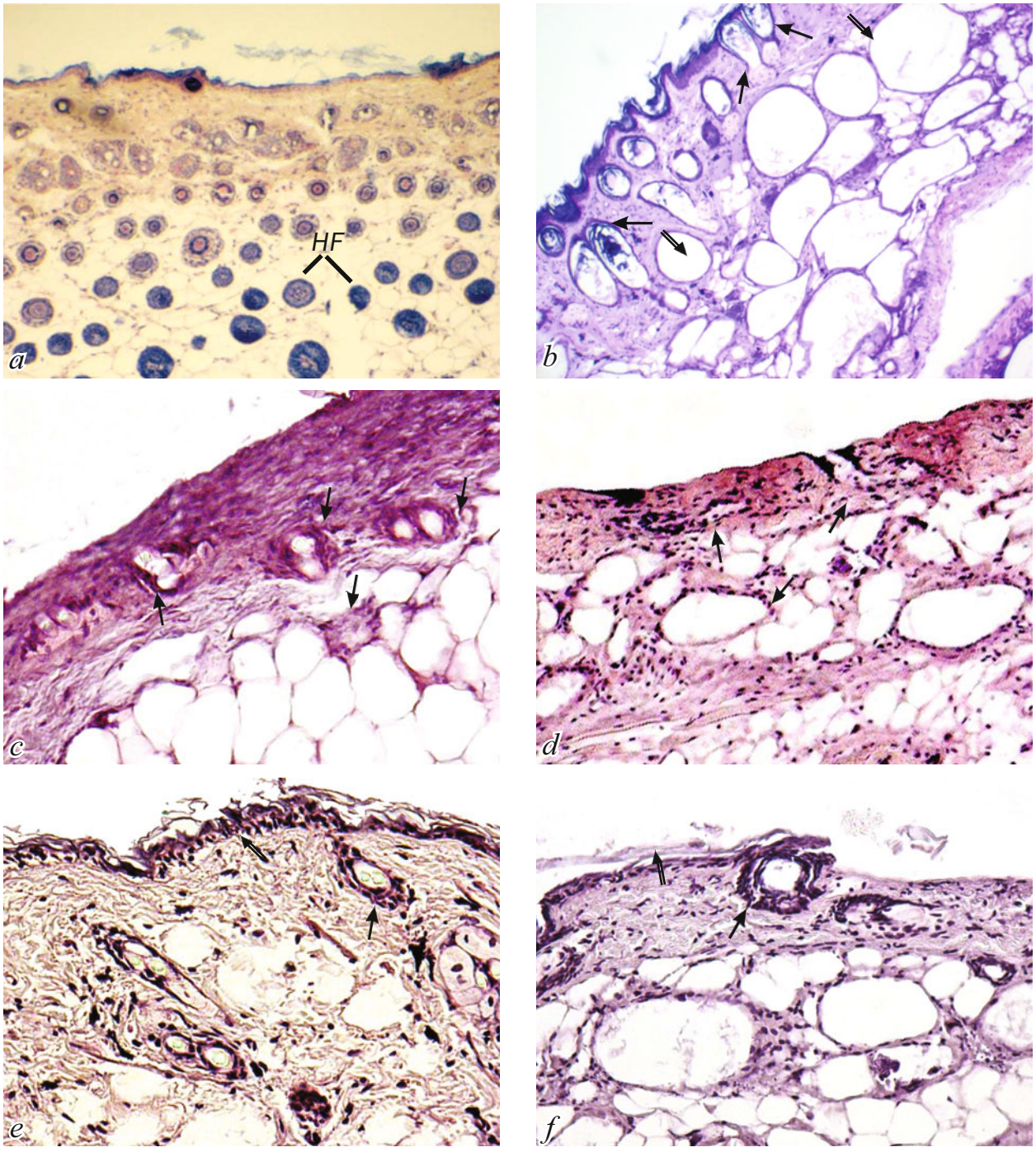

Fig. 1. The skin of intact mice of two groups (*a*, *b*; ×100); the wounds 6 h after infliction of 3A degree burn, spontaneous healing (*c*, *d*; ×200) and after application of a collagen dressing (*e*, *f*; ×200). Hematoxylin and eosin staining. *a*) skin of an outbred albino mouse (cross-sections); *b*) two types of epithelial structures in the derma of mutant Hr^{hr}/Hr^{hr} mouse: sac-like formations (arrows) bound to the epidermis and bubbles of different sizes (double arrows); *c*) burn wound in an outbred mouse: no epidermis, destructive changes in the derma, epithelial cell proliferation in intact HF (arrows); *d*) burn wound in an Hr^{hr}/Hr^{hr} mouse, leukocytic infiltration of the derma, epithelial proliferation of intact "sacs" and dermal cysts (arrows); *e*) active proliferation and migration of HF epithelial cells (arrow), formation of epithelial layer on wound surface (double arrow) in an outbred mouse; *f*) Hr^{hr}/Hr^{hr} mouse: proliferating and migrating epithelial cells of dermal cysts (arrow) line the wound surface with a thin layer (double arrow).

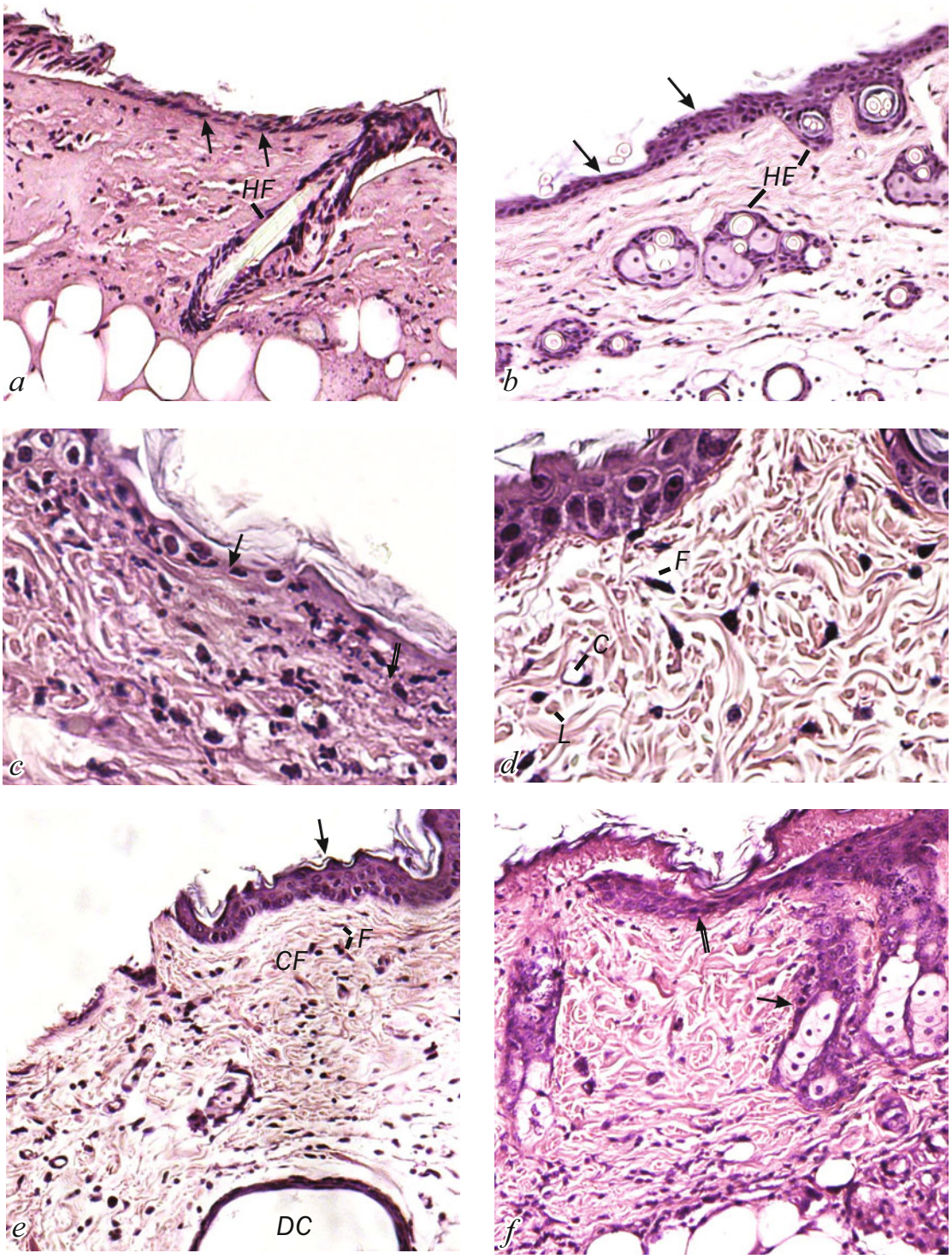

Fig. 2. Burn wounds after 3A degree burns in mice 24 h after the trauma. Hematoxylin and eosin staining. *a*) wound surface in an outbred mouse: HF epithelium proliferation and formation of a thin epithelial layer (arrows; ×200); *b*) the wound healing under biological dressing in an outbred mouse: active proliferation of HF epithelial cells (arrows show the epithelium; ×200); *c*) wound derma in an outbred mouse: destructive changes and edema. Dermal surface on the left (arrow) is covered by a layer of epithelial cells; no epithelium on the right (double arrow); pronounced inflammatory reaction (accumulation of leukocytes and solitary bacterial cells; ×600); *d*) the derma in the wound healing under biological dressing in an outbred mouse: normal bilayer epithelium; *F*: fibroblasts; *L*: leukocytes; *C*: capillaries (×600); *e*) wound surface in an Hr^{hr}/Hr^{hr} mouse: normal bilayer epithelium on the right (arrow), no epithelium on the left; *F*: fibroblasts; *CF*: collagen fibers; *DC*: dermal cyst lined by 2-3 layers of epithelial cells (×200); *f*) wound healing under collagen dressing in an Hr^{hr}/Hr^{hr} mouse: pronounced proliferation of the epithelium in the "sacs", bound to sebaceous glands (arrow). Multilayer epithelium grows under the detaching crust (double arrow; ×200).

lium. Nothing special was observed in the reparative regeneration of the derma. As a rule, the epithelium covered the derma in which no inflammation was detected; solitary fibroblasts and leukocytes were seen among loose collagen fibrils.

Mitoses and even more so results of autoradiographic study attested to proliferative activity of cells. Proliferative activity of the wound cells was recorded in Hr^{hr}/Hr^{hr} mice during the early period of healing in the wounds treated with collagen dressing. High mitotic activity of follicular epithelial cells in the depth of the derma was observed 6 h after burn (Fig. 3, *a*). Several cells in a visual field were in a state of mitosis simultaneously. Incorporation of ^{3}H-thymidine (indicating DNA synthesis) was detected in the epithelium of dermal cysts in the deep dermal layer (Fig. 3, *b*). One day after burn, labeled thymidine incorporation was observed in the vascular wall cells (endotheliocytes and pericytes) and in cells directly adjacent to the vessels. Pronounced destructive changes were seen in a vessel in the subcutaneous fat of the wound, DNA being intensely produced in some of its endothelial cells (Fig. 3, *c*). Judging from ^{3}H-uridine incorporation (RNA synthesis), the protein-producing function of capillary cells (endotheliocytes and pericytes) and of fibroblasts, preadipocytes, and cells of indefinite phenotype was high in the depth of the derma during this period (Fig. 3, *d*).

Hence, wound healing under collagen dressing in mutant Hr^{hr}/Hr^{hr} mice was characterized by high proliferative activity of epithelial cells in the dermal cysts and capillary wall cells (mainly endotheliocytes). Intensive protein-producing function of the wound cells was detected.

No wound cell proliferation was detected by the radioautographic method in the burn wounds healing without biological dressing in mutant mice.

In outbred mice, no incorporation of ^{3}H-thymidine (DNA synthesis) in the wound cells was observed 6 h after burn infliction during wound healing with-

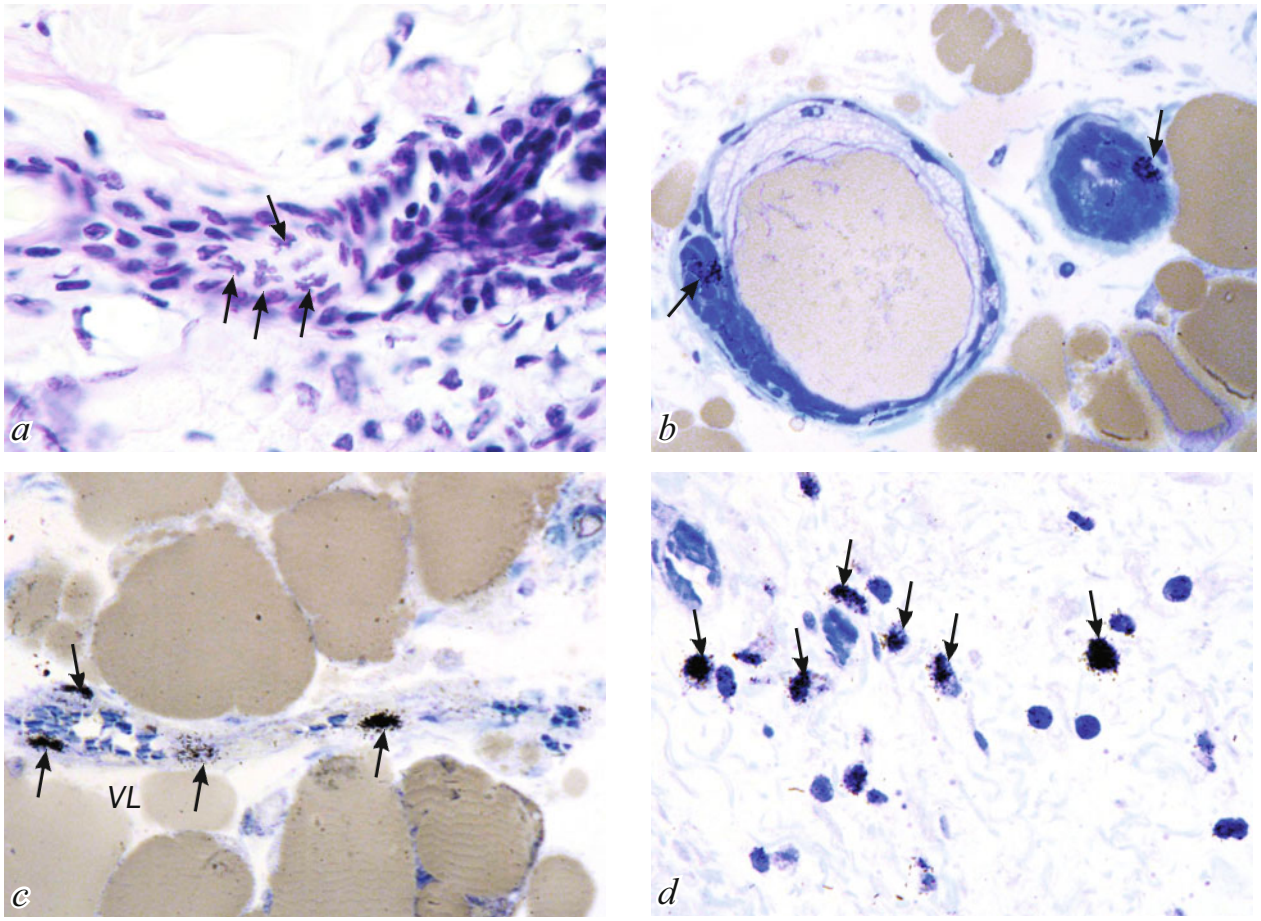

Fig. 3. Proliferative and protein producing activities of cells in 3A degree burn wound in Hr^{hr}/Hr^{hr} mice during the early period of healing under collagen dressing. *a*) high proliferative activity of epithelial cells in HF derivative 6 h after burn. Several cells simultaneously are in a state of mitosis (arrows). Hematoxylin and eosin staining (×630); *b*) ^{3}H-thymidine incorporation (black silver grains) in epithelial cells of two dermal cysts (arrows) in the depth of the wound 6 h after burn infliction; *c*) ^{3}H-thymidine incorporation (black silver grains) in vascular wall endothelial cells in subcutaneous fat 24 h after burn. Sludge erythrocytes (arrow) in vascular lumen (VL); *d*) intense incorporation of ^{3}H uridine (black silver grains) in wound cells (arrows) 24 h after burn. *b-d*: semithin sections, toluidine blue staining (×630).

out dressing. The synthesis of DNA was observed in deeply lying tissues of the wound in solitary cells (preadipocytes) only 24 h after wound infliction. In the wounds treated under collagen dressing, ^{3}H-thymidine incorporation in the vascular endothelium of deep layers of the wound was observed 24 h after the injury in these mice, similarly as in the mutants. However, in contrast to the Hr^{hr}/Hr^{hr} mice, these were just solitary cells with low level of DNA synthesis. Capillary wall cells (endotheliocytes and pericytes) and adjacent cells (mainly fibroblasts) in the subcutaneous fat were intensely labeled with ^{3}H-uridine (RNA synthesis) in the wounds treated with and without collagen dressing. This attests to high functional (protein producing) activity of these cells.

In mice of both strains, burn wounds healing under biological dressing epithelialized on day 3. Spontaneously healing wounds epithelialized after 5-7 days.

Healing of wounds after 3A burns (complete destruction of the epidermis and pronounced destruction of the derma) consists in re-epithelialization of burn surface and regeneration of the derma. Migration of keratinocytes from intact epithelial lining under conditions of derma destruction can start only after formation of the primary cellular matrix during the first days [10]. However, it is assumed that migrating keratinocytes can only partially restore the epidermis. Complete re-epithelialization is possible only at the expense of the SC from the skin appendages (primarily HF).

It seems that one of the mechanisms of the stimulatory effect of collagen dressing on burn wound healing is as follows: exogenous collagen in the dressing acts as the primary extracellular matrix essential for migration of retained keratinocytes.

Proliferative activity of follicular epithelial cells in the derma (mitoses) and in the epithelium of deep dermal cysts (^{3}H-thymidine incorporation reflecting DNA synthesis) detected 6 h after burn infliction in the wounds healing under biological dressing in mutant mice indicates stimulation of SC or progenitor cells in HF derivatives forming as a result of disintegration of HF. After disintegration of HF, the area of protrusion of its outer radical sheath (ORS), which is assumed to be the "tissue niche" of SC in the skin epithelium, is retained in the deep dermal cysts [16]. The detection of a large hair fragment in the depth of the derma of mutant mice can serve as an indirect evidence of the involvement of multipotent SC in the stimulation process. Multipotent SC of the HF protrusion differentiate into cells of the epidermis, sebaceous gland, and matrix, forming the hair [8]. Only stimulation of multipotent SC can initiate both, proliferation of the epidermis and hair growth.

By the present time it is assumed that resident SC located in their tissue niche are stimulated by the microenvironment. In accordance with the hypothesis on the protrusion stimulation [8], SC of the ORS protrusion are stimulated to mitosis only after receiving signals from specialized mesenchymal cells; that is, stimulation takes place at the level of epithelial mesenchymal interrelationships. Collagen-1 is a product of mesenchymal cells, fibroblasts, which perceive signals from collagen fibers by the feedback mechanism during skin wound healing [5]. Presumably, mesenchymal cells react also to signals from collagen, while SC, in turn, perceive its signals. As a result of HF "degradation", the dermal papilla containing the mesenchymal cells is lost in mice with mutation in the Hr gene. This presumably results in "simplification" of the SC stimulation mechanism.

The fact that proliferative activity of HF under conditions of pronounced stimulatory effect of the dressing on wound healing could not be detected in outbred mice by the radioautographic method (in contrast to Hr^{hr}/Hr^{hr} mutants) does not rule out the differences in the mechanisms of SC stimulation in HF ORS protrusions in the studied mouse strains.

Proliferative activity (incorporation of ^{3}H-thymidine, DNA synthesis) in the vascular wall cells (endotheliocytes and pericytes) in the deep layers of the wound was detected 1 day after burn injury in the wounds healing under collagen dressing. This activity was particularly high in endotheliocytes of vessels with destructive changes in Hr^{hr}/Hr^{hr} mutants.

It was hypothesized not once that the vascular wall contains mesenchymal multipotent precursor cells capable of differentiating in different directions, depending on the conditions [4]. This viewpoint was confirmed in many studies [11,15,18]. We previously showed that capillaries in tissue are centers of proliferation and differentiation [2,4]. In addition, some capillary cells can survive the destruction of capillaries, proliferate, and differentiate into tissue cells, for example, fibroblasts. The present study showed that capillary wall cells exhibit not only high proliferative, but also functional (protein-producing) activities. Vascular endotheliocytes and pericytes in the subcutaneous fat and the adjacent cells intensely incorporated ^{3}H-uridine (produce RNA). As we know, RNA synthesis in a cell indicates its functional activity (production of its specific proteins) and reflects production of new proteins, determining the direction of its differentiation.

Hence, it seems that capillary wall contains resident mesenchymal multipotent cells, involved in the regeneration of the derma. It is not clear, which of the vascular wall cells (endotheliocytes or pericytes) possess this activity. Recent reports advocate the role

of pericytes in this process [13,15]. For example, it was proven that endothelial cell plays the key role in connective tissue regeneration and fibrosis [9]. In our study, endotheliocytes most intensely produced DNA (exhibited proliferative capacity). The process of skin revascularization is also important. According to modern concepts, this process in adult life is provided for by the resident endotheliocyte precursors and circulating endotheliocyte precursors of bone marrow origin [13,14].

Bone marrow SC circulating in the blood and infiltrating the wound together with neutrophils are now believed to play an important role in regeneration of the derma [6]. These are the so-called multipotent adult progenitor cells (MAPC).

Hence, the results of our study indicate that the regularities of reparative regeneration of the skin in hairless mice with *Hr* gene mutation are basically the same as in skin regeneration in outbred albino mice. Despite significant changes in the morphology of HF in mutant mice and disorders in its basic function (hair formation), its significance as one of the main sources of epithelial SC retained by its derivatives (dermal cysts) is not lost. Reparative regeneration of the derma in these mice was normal. Moreover, the stimulatory effect of biological dressing was more pronounced in mutant Hr^{hr}/Hr^{hr} mice.

By the present time, we cannot yet present the final experimental results disclosing the mechanisms of the effect of our biological dressing with collagen-1 on reparative regeneration of the skin after burn injury in mice of two genetic groups. However, the fact that this dressing markedly stimulated wound healing is obvious.

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