Mammalian Mechanisms of Wound Healing in Rats with Streptozotocin-Induced Diabetes Mellitus

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Background. Wound healing disorders and formation of diabetic foot, a severe disabling complication of diabetes mellitus, are accompanied by nervous system impairment and/or ischemia.

Objective — the study was aimed at assessing the effect of peripheral innervation disorders on the regulation of tissue repair in the streptozotocin-induced rat model of diabetes mellitus.

Material and methods. The study was carried out in male white outbred rats (n=70). The animals were wounded 42 days after induction of diabetes by injecting streptozotocin (diabetes group; this group received insulin Lевемир at a dose of 2 units/kg in saline subcutaneously to reduce mortality), or after injection of citrate buffer (CB group). Skin samples were taken on day 8, 16, and 24 after wound modeling. Pain sensitivity was assessed in all animals. The resulting skin fragments were fixed, dehydrated, and embedded in paraffin according to standard procedures. Sections were stained with hematoxylin and eosin, antibodies specific for Ki-67, α1, β1, and β2-adrenoreceptors. Reduced density of β2-adrenoreceptors was observed at the areas remote from the wound in CB group rats. Reduced density of β2-adrenoreceptors was observed at the areas remote from the wound in CB group rats. Reduced density of β2-adrenoreceptors was observed at the areas remote from the wound in CB group rats.

Results. Tail withdrawal time measured on day 56 was higher in DM group rats as compared to the control group (p=0.017). CB group demonstrated a tendency towards more rapid wounds healing than diabetic animals, although the difference was not statistically significant due to wide scatter of data in the DM group (p=0.045). The intensity of staining for Ki67 was lower in the DM group (p=0.045). Reduced density of β2-adrenoreceptors was observed at the areas remote from the wound in CB group rats. Reduced density of β2-adrenoreceptors was observed at the areas remote from the wound in CB group rats. Reduced density of β2-adrenoreceptors was observed at the areas remote from the wound in CB group rats. Reduced density of β2-adrenoreceptors was observed at the areas remote from the wound in CB group rats. Reduced density of β2-adrenoreceptors was observed at the areas remote from the wound in CB group rats. Reduced density of β2-adrenoreceptors was observed at the areas remote from the wound in CB group rats.

Conclusion. The results show no correlation between altered innervation and impaired tissue repair in rats with streptozotocin-induced diabetes.

Keywords: peripheral neuropathy, streptozotocin-induced diabetes, tissue repair, nervous regulation, keratinocytes.
Special focus is currently placed on investigating the effect of the nervous system on the key wound healing mechanisms, especially on proliferation and migration of keratinocytes, the main cells of human skin [4].

There are two main factors making the research into the wound healing mechanisms in patients with diabetes mellitus challenging. First, skin has its own nonneuronal catecholaminergic system [7]. Second, keratinocyte response depends on the intracellular mediator system [6–9]. It has been proved in cell culture experiments that β,-adrenergic receptors (β,AP) play a role in regulation of keratinocyte life cycle. These receptors are transmembrane proteins bound to G proteins, which are abundantly expressed in undifferentiated human skin keratinocytes. Whether the intracellular cAMP level will increase or decrease depends on what G-protein α subunit (Gαs or Gαi) is expressed in the cell. Stimulation of β,AP enhances keratinocyte galvanotaxis, migration, proliferation, and differentiation. The presence and impact of other subtypes of adrenergic receptors has been less studied. The data obtained in cells do not take into account the regulatory effects of the organism, changes in the metabolic rate of mediators, nerve ending density, and severity of diabetic neuropathy.

It has been demonstrated that keratinocytes cultured in a glucose-rich medium are characterized by disrupted proliferation and migration (expression of integrin α3, the keratinocyte growth factor, is downregulated). These cells form incomplete gap junctions; the oxidative stress and level of apoptosis are elevated in them. Angiogenesis in tissues surrounding the keratinocytes is disrupted, matrix metalloproteinases are overproduced, and the infection risks are high in patients with diabetes mellitus [10–12]. All these factors contribute to the formation of chronic non-healing wounds. However, we have found no comprehensive studies focused on the effect of neuropathy on wound healing in diabetes mellitus in the available literature.

OBJECTIVE

This study was aimed at investigating the development of neuropathy as a potential mechanism for impaired wound healing in the streptozotocin-induced rat model of diabetes mellitus.

METHODS

The study was carried out in male white outbred rats weighing 350 ± 25 g (n = 70). The animals were kept in the vivarium with controlled illumination (12 hour light/12 hour dark cycle) and free access to food and water. The rats were randomly assigned into groups according to their body weight and heart rate variability.

Diabetes mellitus was simulated using a single intraperitoneal injection of streptozotocin (65 mg/kg) in cold 0.1 M citrate buffer (pH = 4.5, t = +4°C) [3]. Blood glucose level was measured on day 3. The animals with glucose level < 15 mM/mL were removed from the experiment. The day of diabetes mellitus verification was considered day 1 of its development (the DM group). Next, throughout the entire experiments, animals in this group received a subcutaneous supporting injection of insulin detemir (Levemir®) at a dose of 2 units/kg in saline once daily, in the first half of the day. The insulin dose was pre-selected so that the animals would have significant hyperglycemia throughout the day.

Rats given a single intraperitoneal injection of cold 0.1 M citrate buffer (pH = 4.5, t = +4°C) in a proportional volume (CB group, control) were used as a control. Streptozotocin or citrate buffer was injected in the second half of the day, when animals were least active. An additional group of intact animals (the IC group) was also studied.

Blood glucose levels of the rats were measured weekly using an iCheck® blood glucose meter.

Pain sensitivity (the time interval between the moment when animal’s tail tip (2 cm) was submerged in water with a temperature of 55 °C and the time when animals flicked their tails) was measured weekly.

On day 42, round excisional wounds 2 cm in diameter were made below the left shoulder blade using the scissors in the DM and CB groups narcotized with chloral hydrate. After several manipulations, chloral hydrate was replaced with diethyl ether as chloral hydrate caused animals’ death. The wound surface area was measured immediately after wounding and then every 3 days using the Universal Desktop Ruler® software. Skin specimens were collected on days 8, 16, and 24 after wound modeling. In the IC group, skin specimens were collected from 4-month-old animals. At each time point, skin specimens were collected in 10 animals in each group.

The resulting skin fragments were fixed, dehydrated, and embedded in paraffin according to the standard procedure. The samples were cut so that the microspecimen contained the wound defect and the adjacent non-damaged skin. Slices were stained with hematoxylin and eosin according to the standard procedure. Primary anti-rabbit antibodies (Abcam), HRP-conjugated secondary antibodies (goat anti-rabbit, Abcam), and the DAB imaging system were used for immunohistochemical staining to detect Ki67, α, β, and β, adrenergic receptors. The slices were analyzed using a Zeiss Imager A1 Axio light microscope (Zeiss, Germany); the images were obtained using the AxioVision 3.5 software (Zeiss, Germany). The relative staining density was measured using the ImagePro software and compared to the negative control (the specimens stained in the absence of primary antibodies).

Experiment conditions

The experiment was conducted at the Division of Physiology and General Pathology, Faculty of Fundamental Medicine of the Lomonosov Moscow State University.
Ethical review

Experimental animals were handled in compliance with the Order of the Ministry of Health and Social Development of the Russian Federation No. 708n “On Approval of the Rules of Good Laboratory Practice” dated August 23, 2010. Approval for the conduct of experiments was obtained from the Ethics Committee of the Faculty of Fundamental Medicine of the Lomonosov Moscow State University.

Statistical analysis

The Statistica 10.0 and IBM SPSS Statistics 23.0 software packages were used for statistical analysis. The survival was evaluated using the Cox model in which data incompleteness is allowed [2]. Repeated measures ANOVA was employed to evaluate the dynamics of wound healing and changes in pain sensitivity [2]. The expression densities of different proteins were compared using multivariate analysis of variance on a mixed linear model (the Fisher’s Least Significant Difference test). The differences were considered statistically significant at \( p < 0.05 \).

RESULTS

Overall well-being of animals

A single streptozotocin injection caused diabetes mellitus in rats on day 3 post-injection: the blood glucose level was 4–7 times higher than at baseline (6.2 mM) and remained elevated throughout the entire experiment. Animals with diabetes received insulin daily to increase their survival. Three out of 70 rats died during the entire experiment. The body weight of rats at baseline was 367 ± 55 g.

Dynamics of wound healing

There were no differences in the dynamics of wound healing in rats in the DM and CB groups (\( p = 0.672 \)). However, this could be related to a significant variation in wound size in the DM group (Fig. 1). Unlike in the DM group, the wound was completely healed by day 24 in the CB group.

Morphological and immunohistochemical analysis of wound healing

Epidermis of normal thickness, with clearly pronounced basal, spinous, and granular layers, was visualized in skin slices stained with hematoxylin and eosin in intact rats (Fig. 2a).

On day 8 post-wounding, most of the wound surface was closed by granulation tissue intensively infiltrated with inflammatory cells (neutrophils, macrophages, and lymphocytes) (Fig. 2b). The granulation tissue was covered with a layer consisting of necrotic masses. The regenerating epidermis margin was often thickened.

On day 16, the inflammation subsided and signs of acute wound disappeared. Most of the wounds were covered with granulation tissue; epidermis at wound margin was significantly thickened (Fig. 2c). Cricatricial fibrous tissue was formed under the nascent epidermis. Wound re-epithelization was completed by day 16 in some rats in the CB group but not in the DM group.

The fibrous cicatrix lined with a thin epidermis layer was formed on day 24 after wound modeling (Fig. 2d). In some rats in the DM group, complete skin regeneration did not take place during this period.

Immunohistochemical staining

The general view of immunohistochemically stained specimens is shown in Fig. 3. Some keratinocytes in the IC rats largely expressed Ki67, a cell proliferation marker, which corresponds to high regenerative activity of the epidermis. Wounding of rats in the CB group had a negligible short-term effect on regenerative activity. On day 24 of wound healing, when re-epithelization was completed in all rats in this group, expression of Ki67 marker significantly decreased both in the wound margin and in the relatively distant skin regions as compared to the previous time points, to intact animals, and to rats in the DM group (Fig. 4).

In the DM group, Ki67 expression changed significantly at both locations neither on day 8 nor day 16; however, Ki67 expression at wound margin increased on day 24 compared to the IC (\( p = 0.036 \)) and CB groups (\( p = 0.041 \)), as well as to the previous time point (\( p = 0.028 \)).

At most time points, Ki67 expression levels did not differ significantly in the wound center and at a certain distance from it both in the DM and CB groups. Only on day 24, the intensity of Ki67 staining in the wound center in the DM group was higher than that in the distant epidermal region (Fig. 5).

Hence, expression of the proliferation marker at the wound margin in the DM group increased on day 24 post-wounding (\( p = 0.04 \)), being indicative of regeneration intensification. Meanwhile, expression of the marker in the CB group decreased statistically significantly on day 24 as wound re-epithelization was completed by this time (\( p = 0.031 \)).

Epidermis stained with antibodies specific to \( \beta_{1} \)- and \( \alpha_{1} \)-adrenergic receptors was detected in none of the...
groups, including the IC group, which may indicate that these adrenergic receptors are not expressed in rat keratinocytes. On the other hand, $\beta_2$-adrenergic receptors were significantly expressed in keratinocytes in all groups of animals, including the IC group. It should be mentioned that positively stained cells were predominantly located within the basal layer of epidermis (Fig. 2b).

On days 8 and 16, expression of $\beta_2$-adrenergic receptors did not differ between the groups and skin regions. Their expression was downregulated in distant skin regions in the CB group ($p = 0.046$) on day 24 (Fig. 6).

**DISCUSSION**

Neuropathy is one of the possible mechanisms responsible for disruption of wound healing in the streptozotocin-induced rat model of diabetes mellitus.

The results of the pain-sensitivity test demonstrated that rats in the DM group develop peripheral neuropathy that affects sensitive nerve endings during the wound-healing period under study [11,12]. The morphological changes in animals’ skin and the immunohistochemical data
agree with the fact that animals develop neuropathy, so we
can infer that these changes are interrelated.

We observed no statistically significant differences be-
tween the rates of healing of the wound created on day 42
after the rats in the control (CB) and DM groups devel-
oped hyperglycemia. However, the wound size was re-
duced over time, the intragroup variation decreased, and
complete wound healing was observed by day 24 post-
wounding in all rats in the CB group. Animals in the DM
group had both higher and lower wound healing rates;
however, complete wound healing by 24 days post-wound-
ing was observed in none of rats in this group. This fact
increased the intragroup variation, which impeded the de-
tection of differences between the CB and DM groups.
The different wound healing rates in rats with DM can be
caused by differences in high blood glucose level, which
depends on sensitivity of pancreatic beta cells to strepto-
zotocin.

When histological specimens were examined using a
light microscope, the image of acute wound was gradual-
ly replaced with that of wound healing by secondary in-
tention: the leukocyte infiltration was reduced, the wound
healing area was formed, new vessels were formed, and
connective tissue filled the wound. In the CB group,
wound repair was completed on day 24 post-wounding
(day 56 since experiment initiation). All healing stages
were slowed down in the DM group; the wound was not
closed in most of animals in this group by the end of the
experiment.

Ki67 staining revealed the high level of proliferative
activity in skin of intact rats. Wound modeling did not al-
ter the proliferation level in the CB group neither in the
skin area distant from the wound margin nor in the wound
margin. The total proliferation level at the wound site de-
creased when the wound was completely healed. Interest-
ingly, Ki67 expression in the wound margin in DM group
animals with a non-healed defect increased by day 24 af-
ter wound modelling. This result demonstrates that skin
is repaired in healthy rats due to selective changes in pro-
liferative activity of various keratinocyte subtypes: the re-
pair of healthy skin in the deeper skin layers under the thin
layer of epidermis continues as the wound is closed. The
Ki67 of keratinocytes decreases simultaneously. It seems
likely that activation of the population of keratinocytes
involved in wound healing is disrupted in animals with DM,
so the baseline proliferative activity turns out to be insuf-
icient to heal the defect and an increase in the Ki67 level is observed by day 24 post-wounding.

We have not revealed any correlation between diabetes mellitus, the wound healing stages, and adrenergic receptor density in rat epidermis. No significant intergroup differences in staining density of β2-adrenergic receptors in keratinocytes were observed. The reduced receptor expression in skin distant from the wound in CB group rats has not been interpreted yet. Overall, it can be inferred that the density of β2-adrenergic receptors in rats with diabetes mellitus is not changed during wound healing. Nevertheless, further research into this problem is needed to draw any specific conclusions on whether or not peripheral innervation is altered during wound healing in rats with diabetes mellitus. This research needs to include evaluation of the status of other components of the sympathetic and parasympathetic nervous system in the skin.

CONCLUSIONS

This study allowed us to obtain new information about wound healing in the streptozotocin-induced rat model of diabetes mellitus. According to the proposed protocol, rats receiving supporting insulin therapy developed diabetes mellitus that did not cause animal death during 56 days. Pain sensitivity decreased gradually, which was especially clearly seen by day 56 of the experiment. The results of morphological examination demonstrate that wound re-epithelialization was slowed down in rats with DM. In the final point of the study, proliferative activity of keratinocytes in the wound margin increased in these rats, while in the CB group epithelization was completed by this time point and proliferative activity was reduced. Changes in β2-adrenergic receptor density in rats were observed neither after diabetes mellitus was induced nor during wound healing.

Studies focused on innervation density in skin along the wound margin, concentration of neurotransmitter metabolites, and broadening the range of investigated neuronal and non-neuronal systems regulating wound healing will provide an integrated understanding of the processes that take place in chronic diabetic wounds. Furthermore, they will allow one to find novel potential therapy targets and eventually to improve quality of medical aid rendered to patients with diabetic foot syndrome.

SUPPLEMENTARY INFORMATION

Supplementary materials

Figure 2. Morphology of rat skin specimens stained with hematoxylin and eosin.
The figure is available in the full-color inset and online: http://doi.org/10.14341/probl9691-3197

Figure 3. Examples of immunohistochemical staining.
DAB.
The figure is available in the full-color inset and online: http://doi.org/10.14341/probl9691-3198

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Литература

Fig. 2. Morphology of rat skin in specimens stained with hematoxylin and eosin

a — typical histological image of the intact rat skin; b — the thickened margin of regenerating epidermis on day 8 post-wounding; c — the thickened margin of regenerating epidermis on day 16 post-wounding; d — cicatricial tissue and regenerated epidermis within the wound on day 24. 1 — granulation tissue; 2 — necrotic masses; 3 — wound margin; and 4 — fibrous tissue.

Fig. 3. Examples of immunohistochemical staining.

DAB: a — Negative control: only a strip of the stratum corneum is stained nonspecifically; b — staining with antibodies specific to β2 adrenergic receptors, wound margin, stained cells predominantly localize in the deep epidermal layers; c — staining with antibodies specific to Ki67, wound margin.