In both developed and developing countries around the world, changes in lifestyle have resulted in a dramatic increase in the incidence of chronic disease, particularly diabetes mellitus. The number of people suffering from diabetes mellitus is predicted to increase from the current estimate of 150 million to 220 million in 2010, and then to 300 million by 2025. In the clinical setting, patients with diabetes have a higher risk of developing pressure ulcers than people without diabetes. Patients with diabetes also often have delayed wound healing, which results in a prolonged treatment peri- 

Ulceration and Delayed Healing Following Pressure Loading in Hyperglycemic Rats With an Immature Dermal Collagen Fiber Network

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Abstract: The collagen fiber network plays a key role regarding mechanical properties that maintain tissue shape, absorb stress, and recover from tissue deformation. The purpose of this study was to reveal the effects of hyperglycemia on the process of ulceration and wound healing, and on the structure of dermal collagen network.

Methods. A spontaneous type 2 diabetic, non-obese rat without hyperlipidemia (GK rat) was used. On the right abdominal flank region, 8 kg/3 cm² of pressure was loaded, and then the morphological change in wound area was macroscopically observed. The tissue of wounded area and healthy area on opposite side of the abdomen was collected and histologically analyzed on days 3, 5, 7, and 14 after wounding.

Results. The hyperglycemic animals showed severer ulceration and delayed wound healing after pressure loading compared to control rats. The diabetic rat had an immature collagen fiber network with poor cross-linkage in the dermis. In the wounded area, collagen fibrils were packed more densely and reconstruction of the fiber network was delayed.

Conclusion. These results suggest that the disrupted structure of the collagen network lowers the tolerance of diabetic skin to external pressure loading, and that this delayed reconstruction increases time to healing.

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od. This phenomenon has become a significant problem that negatively affects quality of life for patients with diabetes and increases medical costs.

Generally, pressure ulcers are caused by a pressure force greater than capillary pressure, which thus induces tissue hypoxia, cell deformation, reperfusion, and ultimately, cell death. Collagen plays a key role in the mechanical properties acting to maintain tissue shape, absorb stress, and recover from tissue deformation. Collagen has a triple-strand coiled structure, which is termed tropocollagen. An aggregate of tropocollagen forms fibrils and bundled fibrils that develop into the thick, rope-like structure of collagen fiber. Extensive pressure loading results in irreversible changes in the structure of the collagen fiber network. Aaro et al reported the densely packed fibrils in the boundary area and the disappearance of fibrous structure in the damaged area in comparison to the healthy area. The collagen network also plays various important roles, such as interacting with platelets and fibronectin, and forming a scaffold for fibroblastic proliferation during the wound healing process.

The relationship between diabetes mellitus and cutaneous wound healing has been widely investigated. Loots et al reported that diabetic foot ulcer-derived dermal fibroblast showed delayed cell growth and abnormal morphology, a large dilated endoplasmic reticulum, and multiple lamellar and vesicular bodies. In a wound healing model, the volume of deposited collagen was also reported to significantly decrease in the ob/ob mouse. An in-vitro study revealed that dermal fibroblasts derived from diabetic animals showed reduced collagen synthesis, a dysfunction in migration, vascular endothelial growth factor production, and a response to hypoxia. The collagen volume was observed to be significantly low in the streptozotocin induced-diabetic rat skin in comparison to the control rat. Furthermore, patients suffering from insulin-dependent diabetes mellitus showed alterations in collagen fibers. Based on these facts, hyperglycemia induces cellular dysfunction in dermal fibroblasts, which leads to decreased collagen production and might cause such tissue to develop pressure ulcers; however, since previous studies tended to use in vitro or animal studies including various confounders besides hyperglycemia, the dependent effects of hyperglycemia on the wound healing of pressure ulcers therefore remain to be fully elucidated.

The authors proposed the Goto-Kakizaki (GK) rats as a possible animal model to examine the effect of hyperglycemia alone on wound healing because this spontaneous diabetes mellitus rat showed increased slight hyperglycemia from 8 weeks after birth without obesity or hyperlipidemia. The present study researched the immature structure of the dermal collagen fiber network, severe ulcer development, and delayed healing following pressure loading in the GK rats compared to normal rats.

**Materials and Methods**

**Animals.** Spontaneous type 2 diabetic rats (GK/slc, Sankyo Laboratory Service, Tokyo, Japan) were used. The rats were produced as a result of selective breeding over many generations of nondiabetic Wistar rats with glucose intolerance by Goto and Kakizaki. Twenty-two male GK rats age 12–14 weeks old were used as the hyperglycemic group, and 15 male Wistar rats age 12–14 weeks old served as the control group. Four rats from each group were used for electron microscopic observations. All rats were individually caged in an air-conditioned room with standard diet ad libitum. They were exposed to 12 hours light and 12 hours darkness.

The animal ethics committee of The University of Tokyo and Kanazawa University approved the study. All animals were treated according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH).

**Wounding procedures.** The method of wounding was adopted from the modality described in a previous study on ischemic wounds. To ensure the reproducibility, the wounds were experimentally created on the flank regions where vertical pressure can be applied for a set period of time with a degree of stability. After weighing and anesthetizing each rat by the intraperitoneal administration of sodium pentobarbital (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan) of 30-mg/kg bodyweight, blood was taken from tale vein to examine blood glucose with a glucose- monitoring device (GT-1641, Arkray, Kyoto, Japan). Thereafter, they were shaved on the right flank region with electric clippers. Two 2-cm incisions, 5 cm–6 cm apart that extended to the peritoneum were then made in the right flank region using an electrocautery scalpel. A 2-cm wide metal plate was inserted from one incision and then it exited from the other. The rat and metal plate were then fixed to the experimental device. Eight kilograms pressure on a 3 cm² area was applied for a total of 6 hours. After relieving the pressure, the metal plate was removed and the incisions were closed with non-absorbable nylon sutures at 5-mm intervals.

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Subsequently, the wounds were covered with hydrocolloid dressing (Tegasorb™, 3M, St. Paul, MN) to prevent scabbing due to drying.

The blood glucose levels were examined every day. We macroscopically observed the wounds every day to confirm whether there was any change in the wound color or ulceration. The wound area was measured with a planimeter (PLANIX 108, Tamaya Technics, Tokyo, Japan).

**Histological observations.** The rats were sacrificed under deep anesthesia on days 1, 3, 5, 7, and 14. The tissue specimens including the wound area and the surrounding area of the wound were then excised. Tissues without pressure loading were also excised at the same time. Tissue samples were fixated in 4% paraformaldehyde diluted with 0.1 M phosphate buffer (pH 7.4) for light microscopic observations. Thereafter, for electron microscopic observations, some tissue samples were fixated in phosphate buffer containing 2% glutaraldehyde and 4% paraformaldehyde.

After fixation, light microscopic samples were dehydrated in a graded series of ethanol and embedded in paraffin. The paraffin serial sections (5-µm thick) were cut and stained with hematoxylin and cosin (H&E), and Azan staining.

For scanning electron microscopic (SEM) observations, the samples were washed with 0.1 M phosphate buffer with 5-minute intervals for three times after fixation, and then were post-fixed with 2% osmium tetroxide in the same buffer for 2 hours. They were then dehydrated in a series of ethanol (70%, 95%, 100%, 100%, and 100%). The ethanol was cleared by t-butyl alcohol. The samples were dried using a Freeze Dryer (ES-2030, Hitachi High-Technologies, Tokyo, Japan), were vapor deposited by osmium, and then observed with a field emission scanning electron microscope (S-4200, Hitachi High-Technologies).

**Statistical Analysis**

All data were expressed as the means ± SD. Differences between the two groups were evaluated by either the t-test or the Wilcoxon rank sum test. A mixed effect model with Dunnett correction was used to assess the significance of difference between the two groups for each time point. Kaplan-Meier curves were used to assess the time from the start of study treatment to the time of reporting of healing events and comparisons were made with the log rank test. \( P < 0.05 \) was considered statistically significant. All statistical analyses were performed using the Statistical Analysis System software program Ver. 9.1 (SAS Institute, Cary, NC).

**Results**

**Weight and blood glucose.** The body weights were 350.2 g ± 13.3 g in the control group and 294.5 g ± 18.6 g in the hyperglycemic group before wounding (\( P < 0.001 \)), and until day 5 they decreased 50 g in both groups and then became stable. Before wounding blood glucose was 112.7 mg/dL ± 22.8 mg/dL in the control group, and 209.2 mg/dL ± 44.0 mg/dL in the hyperglycemic group (\( P < 0.001 \)). On day 14, the end of the observations, the blood glucose level was 102.0 mg/dL ± 24.0 mg/dL in the control group, while 214.0 mg/dL ± 71.0 mg/dL in the hyperglycemic group (\( P = 0.007 \), Figure 1). The blood glucose in the hyperglycemic group was significantly higher throughout the study period.

**Macroscopic findings after wounding.** Immediately after pressure unloading, a dark red circle matching the wound area with edema at the circumference was seen to a closely similar degree in both the hyperglycemic group as well as the control group (Figure 2A–I). In the control group, on day 3, a shallow wound with a denuded epidermis was seen, and no full-thickness ulceration was observed (Figure 2C). All six rats healed by day 11. Conversely, in the hyperglycemic group, a partial epidermis deficit was also detected on day 3 (Figure 2K). Thereafter, a shallow ulceration was confirmed by day 7 (Figure 2M), which later developed into a deep ulcer. Epithelialization did not advance and only two of seven rats healed by day 14. Furthermore, in...
the hyperglycemic group, there was one rat that did not heal by day 93. The Kaplan-Meier survival curve revealed the decrease in the healing rate in the hyperglycemic group (Logrank test; P < 0.001).

**Light microscopic findings.** Leading up to day 3, similar tissue damage, partial epidermal deficits, partial damages of the panniculus carnosus muscle and abdominal muscle, similar inflammation, and infiltration of polymorphonuclear (PMN) cells and macrophages were observed in both the control and hyperglycemic groups (data not shown).

In the control group, the inflammatory reaction was gradually decreased. Migration of epithelial cells was observed on day 5. Epithelialization was complete on day 14 (Figure 3A–C). Azan staining showed the reconstruction of dermal collagen fiber network during healing period (Figure 3G–I).

Conversely, in the hyperglycemic group, HE staining indicated the progression of the degeneration of dermis and the ulcer formation. The infiltration of PMN cells throughout all layers of tissue had drastically increased on day 7 and remained severe even by day 14. The migration of epithelium cells had barely been observed on day 14 (Figure 3D–F). The collagen network degenerated until day 7. The fibrous collagen was slightly increased on day 14, although the density of collagen was remarkably low.

**SEM findings of collagen fibers in the dermis.** In the uncompressed area, although bundled structures of collagen fibers were observed, they did not cross each other in as complicated a manner as that observed in the normal dermis (Figure 4A, C). In the wound area, no thick bundled structures of the collagen fibers were observed in both the control and hyperglycemic samples. The fibrils were packed so densely that it is difficult to identify the individual fibrils in hyperglycemic group (Figure 4B, D).

**Discussion**

We created a severe pressure-induced ulcer with delayed healing in spontaneous diabetic rats, which showed hyperglycemia without microangiopathy. In the control animals, compressed area healed without full-thickness tissue loss, showing only a partial epidermis loss. On the other hand, the hyperglycemic group showed deep ulcerations reaching to the cutaneous muscle under the same loading conditions. In addition, the wounds of the control group showed complete epithelialization and then healed until post-wounding day 11, whereas the hyperglycemic group showed delayed healing with the presence of deep ulceration and uncompleted epithelialization up to day 14. Microscopic and electron-microscopic observations indicated the immature collagen fiber network with poor cross-linkage in diabetic rats. These results suggested that a structural disruption of the collagen network in the hyperglycemic rat decreased the resistance of the dermal layer against any external force, thus resulting in more severe ulceration than that observed in normal animals.

Several studies have so far indicated that the hyperglycemic condition inhibit the proliferation, migration, and cellular dysfunction of dermal fibroblasts and...
Figure 3. Histology of wound area. Hematoxylin and eosin (HE) staining (upper panels) showed severe ulceration and inflammation, and azan staining (lower panels) showed a disruption and poor recovery of the collagen fiber network in the hyperglycemic (HG) group in comparison to the control group.
Magnification: x20
Scale bar = 200 m
The delayed healing including reconstruction of collagen layer and epithelialization might be caused by these inhibiting effects. Furthermore, this study showed that a high concentration of serum glucose induced not only the quantitative alteration, but also structural change of collagen network. Hyperglycemia may therefore induce a qualitative alteration in skin tissue, and elucidating this mechanism of action therefore requires further study.

In patients with diabetes, microangiopathy and abnormal hemorheology also occur due to long-term chronic hyperglycemia. Additionally, the glycosylation of hemoglobin is known to result in less oxygen release into the tissues. The combination of these alterations induces both tissue ischemia and a reduced capillary pressure. The interaction of hematical abnormalities and a disrupted structure of the collagen network could therefore increase the risk of developing pressure ulcers in diabetic patients.

Several previous studies have reported the use of excised full-thickness wound model for investigating the diabetic wound healing. Michael et al showed a delayed wound healing rate with decreased collagen concentration in the diabetic wounds when compared with the control wound over time. Others have indicated that the formation of granulation tissue was dramatically impaired due to reduced fibroblast proliferation, lower levels of collagen deposition, and wound contraction. However, the ultrastructure study on the collagen network in diabetic granulation tissue have not been reported. Since the healing process includes the digestion of damaged collagen fiber and the reconstruction of new collagen network, our results from the pressure ulcer model cannot fully provide the accurate mechanisms of delayed healing in diabetes. In order to develop a novel methodology to promote wound healing in diabetic patients, further studies on the activity of collagen degrading enzyme are required. Some potential investigations might include matrix metalloproteinases and their inhibitors in a pressure ulcer model and the reconstructing ability of collagen network in a full-thickness cutaneous wound model.

In addition to the immature collagen, more severe inflammation cells in the granulation tissue of the diabetic ulcers were found. This is consistent with the previous work, which showed severe and persistent inflammation infiltration. Upchurch et al demonstrated that patients with diabetes who have a foot ulcer had increased levels of CRP compared with diabetic patients without a foot ulcer and normal control patients, suggesting the occurrence of an acute phase response. Future studies are needed in order to clarify the relationship among the structure of collagen network, the local inflammation and the systemic inflammation status in diabetic wounds. As a result of such further studies we can then provide an interesting and impor-
tant knowledge to elucidate the mechanisms of delayed healing in diabetes.

The present study showed the structural abnormality of the collagen fiber network due to hyperglycemia. Since this study was performed using a particular animal model, it is difficult to generalize the results. However, the results have clinical implications, namely that it is important to relieve skin pressure in patients with diabetes because their skin has a low tolerance for external force and limited ability to repair such damage.

Conclusion
The spontaneous type 2 diabetic model rats without obesity and hyperlipidemia showed severer ulceration and delayed wound healing after pressure loading in comparison to the control rats. The diabetic rats had an immature collagen fiber network with poor cross-linkage in the dermis. Within the wound area, collagen fibrils were packed more densely and the reconstruction of fiber network was delayed. These results suggested that the disrupted structure of the collagen network resulted in the low tolerance of diabetic skin for external pressure loading.

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References


