Characteristics of the Epidermis and Stratum Corneum of Hairless Mice with Experimentally Induced Diabetes Mellitus

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Diabetes mellitus induces many pathophysiologic changes in the skin. Even so, dermatologists still lack an animal model of diabetes that enables the direct evaluation of the various functional properties of the skin. Our group induced two types of an experimental type 1 diabetes model in hairless mice by administering either streptozotocin or alloxan, in order to examine the properties of the stratum corneum and epidermis of these animals. The plasma glucose concentrations of the mice at 3 wk after their i.v. injection were significantly higher than those of control mice (streptozotocin, 3.2-fold; alloxan, 3.7-fold). The stratum corneum water content was significantly reduced in both types of diabetic mice, whereas the transepidermal water loss remained unchanged. The amino acid content with normal epidermal profilaggrin processing was either normal or elevated in the stratum corneum of the streptozotocin-treated mice. In contrast, the stratum corneum triglyceride content in the streptozotocin-treated mice was significantly lower than the control level, even though the levels of ceramides, cholesterols, and fatty acids in the stratum corneum were all higher than the control levels. The streptozotocin-treated group also exhibited decreases in basal cell proliferation and epidermal DNA content linked with an increase in the number of corneocyte layers in the stratum corneum, suggesting that the rates of epidermal and stratum corneum turnover were slower in the streptozotocin-treated animals than in the normal controls. In contrast, there were no remarkable changes in any of the epidermal differentiation marker proteins examined. This finding in diabetic mice, namely, reduction in both the epidermal proliferation and stratum corneum water content without any accompanying impairment in the stratum corneum barrier function, is similar to that found in aged human skin. Our new animal model of diabetes will be useful for the study of diabetic dermopathy as well as the mechanisms of stratum corneum moisturization. *Key words: aging/horny layer/insulin/loricrin. J Invest Dermatol 120:79–85, 2003*

Diabetes mellitus induces various forms of dermopathy, for example, bullosis diabeticorum, necrobiosis lipoidica diabeticorum, scleredema diabeticorum, and acanthosis nigricans (Jelinek, 1993). All of these skin conditions are derived from an impaired skin homeostasis in the dermis and epidermis thought to be caused either by diabetes-induced abnormalities of the metabolism or by diabetic complications, such as vasculopathy and neuropathy. Moreover, the insulin resistance and hyperglycemia in diabetes have a confirmed involvement in the impaired functions observed in various tissues of diabetic subjects. Hyperglycemia induces cellular abnormalities via nonenzymatic glycation processes, oxidative-reductive stress, aldose-reductase activation, diacylglycerol-phosphate kinase C (PKC) activation, etc. (Koya and King, 1998; Nishikawa *et al*, 2000). In the case of the skin, diabetes is reported to induce advanced glycosylation end products in the collagen of the dermis (Kennedy and Baynes, 1984; Sternberg *et al*, 1985), and these end products are postulated to produce the characteristic stiffness (Aoki *et al*, 1993; Nikkels Tassoudji *et al*, 1996) and thickness (Collier *et al*, 1989; Forst *et al*, 1994) of diabetic skin, as well as the delayed wound healing observed in the disease (Brown *et al*, 1994; Franzen and Roberg, 1995; Bitar, 1998). Whereas no studies have been conducted to identify the effects of hyperglycemia on the epidermis, the importance of insulin as an essential growth factor for cultured keratinocytes and its substantial influence on their proliferation (Tsao *et al*, 1982) and migration (Ando and Jensen, 1993; Benoliel *et al*, 1997) suggest that the inhibited keratinocyte proliferation in diabetic patients is an important factor in their delayed wound healing. In a more recent study, Wertheimer *et al* (2000) demonstrated that insulin regulates the keratinocyte differentiation. Nerve growth factor is also involved in keratinocyte proliferation, and epidermal basal cells in diabetic skin exhibit depressed genetic expression of nerve growth factor in diabetes (Anand *et al*, 1996). Interestingly, the surface area of corneocytes has been reported to be larger in diabetic patients than in normal individuals (Yajima *et al*, 1991), suggesting that diabetes mellitus may influence the proliferation and differentiation of keratinocytes. Little is known, however, about the functional properties of the stratum corneum (SC), the end product of the epidermis, in diabetes. Efforts to explore this have been hampered by the complicated influences of age on the changes of the SC in diabetic patients.

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Abbreviations: PCNA, proliferating cell nuclear antigen; SC, stratum corneum; TEWL, transepidermal water loss.

For example, it is difficult to distinguish between the pruritus observed in diabetes and that noted in senile xerosis by a simple clinical observation (Jelinek, 1993).

In this study, we produced a new model of diabetic hairless mice using streptozotocin or alloxan, agents well known to destroy the insulin-secreting pancreatic β cells (Wilson and Leiter, 1990). We selected hairless mice because the presence of hair and the hair cycle are known to disturb the functional and biochemical analyses of the SC. Streptozotocin-treated animals are widely used as a model of type I diabetes that exhibits many of the features seen in human patients with uncontrolled diabetes mellitus, including hyperglycemia, polydipsia, and polyuria (Tomlinson *et al*, 1992; Cheta, 1998).

After inducing diabetes in the hairless mice using streptozotocin or alloxan, we evaluated the hydration state and barrier function of the SC. In the streptozotocin group, we measured the changes of the SC lipids and soluble amino acid content in the SC. We found that the diabetic mice had impaired water homeostasis in the SC without any damage in the barrier function. The SC amino acid and epidermal filaggrin levels were normal, but the SC triglyceride levels were significantly lower than those measured in controls. In addition, the epidermal turnover seemed to be retarded in the streptozotocin group. Our experimental results suggested that SC hydration in the streptozotocin-induced diabetic hairless mice may have been regulated by factors other than intercellular lipids and amino acid levels (Horii *et al*, 1989; Imokawa *et al*, 1991b), the major factors heretofore confirmed to play a part in SC hydration. We confirmed that the experimentally induced diabetic hairless mouse model is useful for the study of not only the diabetic dermopathy, but also the mechanism of SC hydration.

MATERIALS AND METHODS

Materials Male hairless mice (Hos/HR-1) were obtained from Hoshino Laboratory Animals (Saitama, Japan). Streptozotocin was purchased from Sigma (St Louis, MO). Alloxan monohydrate was purchased from Wako (Osaka, Japan). Polyclonal antibodies against mouse filaggrin (AF 111), keratin 1 (AF 109), keratin 5 (AF 138), and loricrin (AF 62) were purchased from the Berkeley Antibody Company (Richmond, CA). Monoclonal antibody against cytokeratin 10 (RKSE 60) was purchased from MONOSAN (AM Uden, the Netherlands).

Induction of diabetes mellitus Male hairless mice (7 wk) were divided into two groups: normal control mice and mice with experimentally induced diabetes. Diabetes was induced in the streptozotocin group by a single i.v. injection of streptozotocin (150 mg per kg of body weight) dissolved in 50 mM citrate, pH 4.7. Diabetes was induced in the alloxan group by a single i.v. injection of alloxan (45 mg per kg of body weight) dissolved in 50 mM citrate, pH 4.7. Control mice were injected with citrate buffer alone. The glucose content and transaminase (glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase) activities, markers for disorganization in blood plasma, were measured with a Glucose C II U test kit (Wako, Osaka, Japan) and Transaminase C II U test kit (Wako), respectively, according to the manufacturer's instructions.

Measurements of SC hydration and transepidermal water loss (TEWL) The state of SC hydration was determined by high-frequency measurements of skin conductance. The skin conductance was determined as the average values of five measurements taken with an impedance meter (Skincon-200, IBS Co., Ltd, Hamamatsu, Japan) (Tagami *et al*, 1980). TEWL was measured with a HYDROGRAPH (AMU-100, K and S, Aichi, Japan), as described previously (Haratake *et al*, 1997).

Lipid content in the SC Punch biopsy skin specimens (diameter, 18 mm) were heat-split at 60°C for 30 s to remove the intact epidermis. The SC was isolated by floating the epidermal sheets, basal side downward, on 0.5% trypsin in a phosphate-buffered saline (PBS) for 2 h, and then washing the sheets three times with PBS. SC lipids were extracted by the method of Bligh and Dyer (1959), dried, weighed, and quantified by fractionation using high-performance thin layer chromatography followed by charring and scanning densitometry. Lipids were resolubilized in chloroform/methanol (2 : 1 vol) and spotted on high-performance thin layer chromatography plates (Merck, Darmstadt, Germany). For the analysis of sebum-derived lipids, the plates were developed once in benzene/hexane (1 : 1 vol) to 40 mm, then once in hexane/diethyl ether/acetic acid (70 : 30 : 1 vol) to 80 mm. For measurement of ceramides, the plates were developed once in chloroform/methanol/water (80 : 20 : 2 vol) to 40 mm, once in chloroform/methanol/acetic acid (96 : 3.5 : 0.5 vol) to 64 mm, and once in hexane/diethyl ether/acetic acid (70 : 30 : 1 vol) to 80 mm. After development, the plates were dipped in a charring solution [distilled water containing 10% (w/v) copper sulfate and 8% (w/v) phosphoric acid], dried, and incubated at 180°C for 15 min.The plates were scanned and quantitated using a densitometer (CS9300, Shimazu, Kyoto, Japan).

Oil red O staining Tissues fixed in formalin were dehydrated by a sucrose solution, frozen, and sectioned. The frozen sections (6 μm) were washed in water for 5 min and stained for 30 min (37°C) with 1.8% (w/v) oil red O solution [60% (v/v) isopropanol]. The sections were then washed in water for another 5 min and counterstained for 2 min with hematoxylin.

Amino acid content in the SC SC sheets (2 cm × 2 cm) were stripped from the dorsal parts of the animals with 20 μl cyanoacrylate (Toa Kasei, Tokyo, Japan) and OHP film sheet. Each SC sheet was divided into two equal pieces (1 cm × 2 cm) for quantification of SC amino acids and weight. The sheets were extracted with 10 mM HCl by shaking (16 h) at room temperature, and the extracts were applied to an amino acid analyzer (L-8800, Hitachi, Tokyo, Japan) programmed to detect aspartic acid, threonine, serine, asparagine, glutamic acid, glutamine, glycine, alanine, citrulline, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, ornithine, lysine, histidine, and arginine. The SC weight was determined as described by Imokawa *et al* (1991a). Briefly, the SC sheets (1 cm × 2 cm) were treated with dimethyl formamide under ultrasonication for 30 min to solubilize cyanoacrylate resin. Dispersed SC was separated through filtration with a Millipore filter (Millex-FH, Bedford, MA). The filters with SC were washed with dimethyl formamide and methanol, dried at 40°C for 2 d, and weighed.

Immunoblotting The epidermis was homogenized in a lysis buffer [0.1 M Tris hydroxymethyl aminomethane–HCl (pH 9), 9 M urea, 1% 2-mercaptoethanol, 1% sodium dodecyl sulfate, 1 mM ethylenediamine tetraacetic acid, and 0.1 mM phenylmethanesulfonyl fluoride] and centrifuged (15,000 × *g*, 15 min). The supernatant was applied to immunoblotting.

The SC sheet stripped from the animal with cyanoacrylate (Toa Kasei, Tokyo, Japan) was treated with dimethyl formamide to solubilize cyanoacrylate resin, separated by centrifugation, and washed with PBS. The SC extract was prepared with the lysis buffer. Buffer (2 μg per lane for filaggrin or 20 μg per lane for loricrin) was applied to electrophoresis on a 5–13% gradient sodium dodecyl sulfate–polyacrylamide gel, then transferred electrophoretically to a polyvinylidene fluoride membrane (Polyscreen, NEN Life Science Products, Inc., Boston, MA). After blocking of the nonspecific binding sites with 5% skim milk in PBS containing 0.1% Tween 20, the membrane was incubated with corresponding antibodies in the blocking buffer for 2 h at room temperature followed by washings with PBS containing 0.5% Tween 20 (PBST). Next, the membrane was incubated with the peroxidase-conjugated goat anti-mouse IgG (H + L) antibody (ICN Biomedicals, Inc., Aurora, OH) or the peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (Dako Japan Co., Ltd, Kyoto, Japan) for 1 h. After washing with PBST, the membrane was visualized using an enhanced chemiluminescence kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL). The concentrations of used primary antibodies were 1 : 10,000 (AF 111), 1 : 400,000 (AF 109), 1 : 100,000 (AF 138), 1 : 1000 (AF 62), and 1 : 500 (RKSE 60) for filaggrin, keratin 1, keratin 5, loricrin, and keratin 10, respectively. The protein content in the epidermis and SC was measured with a DC Protein Assay kit (Bio-Rad Laboratories Inc., Richmond, CA).

Counting for proliferating cell nuclear antigen (PCNA)-positive basal cells in the epidermis Skin specimens were fixed in 10% formalin for 3 d, embedded in paraffin, and cut in consecutive sections measuring 3 μm in thickness. The sections were deparaffinized in xylene and rehydrated in a series of graded alcohols. After treatment with 3% hydrogen peroxide, the sections were stained by an Auto Probe II kit (Biomeda, CA) using anti-PCNA antibody (Biomeda, Foster City, CA). PCNA-positive basal cells and total basal cells (200 cells per slide) were counted.

Epidermal DNA content Epidermal DNA was extracted as described by Moore (1999). Briefly, epidermis prepared by the above method was digested with proteinase K (0.2 mg per ml, 50°C, 16 h) and extracted with a phenol/chloroform/isoamyl alcohol solution (three times) to remove protein contaminations. Next, the DNA solution was digested with RNAase A (10 μg per ml, 37°C, 2 h), extracted again with a phenol/chloroform/isoamyl alcohol solution, precipitated with 100% ethanol, and

washed with 70% ethanol. Concentrated DNA was re-solubilized with water and quantified by measurement of the ultraviolet absorption.

Measurements of the corneocyte surface area Corneocytes were collected by pressing a piece of low-strength adhesive tape (Suhada Checker, Kanebo, Tokyo, Japan) against the skin. Tapes with corneocytes were incubated in an eosin solution, washed with distilled water, and dried. Microscopic images of 20 stained corneocytes were traced, and their areas were binarized and measured by an image analyzer (Nexus 6800, Nexus, Tokyo, Japan). The average surface area of 20 corneocytes was used as a representative value for each mouse.

Counting the number of corneocyte layers The skin samples were quickly frozen and sliced into 6 μm thick cryostat sections. The sections were stained with a 1% aqueous solution of safranin for 1 min and flooded with a 2% KOH solution by the method described by Ya Xian *et al* (1999). Safranin produces a clear reddened intensification of the intercellular portions of the SC even in the KOH solution. The number of swollen corneocyte layers was counted at 10 locations per section, avoiding the sites of follicular ostia, to obtain a mean.

Statistics Obtained values were represented as the mean with SEM. Probability levels of $p < 0.05$ and $p < 0.01$ were considered significant when the results were analyzed by the Student's t test.

RESULTS

Induction of diabetes mellitus with streptozotocin and alloxan All the mice developed diabetes rapidly after the injections of streptozotocin 150 mg per kg (**Table I**). A significant increase in the blood glucose concentration was noted from the second day after the injection, and the increase continued time-dependently up to 3 wk. After 4 wk, the transaminase activity of the diabetic mice was similar to that of the controls. There was no increase in body weight during the 3 wk period of streptozotocin induction (data not shown). The injection with alloxan (45 mg per kg) also induced the rapid development of diabetes with a significant increase in blood glucose concentration observed from the second day after the injection. The transaminase activity (glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase), a marker for liver damage, remained normal in the alloxan-treated mice for the first 2 wk, then rose above the control level after week 3. This latter finding indicated that alloxan was more toxic than streptozotocin.

Changes in functional properties of the SC The streptozotocin-treated mice showed a time-dependent decrease in the high-frequency conductance of the SC compared with the control group after the induction of diabetes (**Fig 1*A*,*C***). In contrast, there was no difference in TEWL between the control and streptozotocin groups during this induction phase (3 wk) (**Fig 1*B*,*D***). At 3 wk after the injection, high-frequency conductance of the SC of the diabetic animals was half that of the control group (**Fig 1*C***). At this point, results of freeze-dry weighing confirmed that the water content of the whole skin was no lower in the streptozotocin group than in the control group (control, $69.0 \pm 0.8\%$; streptozotocin, $71.3 \pm 0.5\%$; $n = 5$), suggesting that the viable skin tissue in the former was not dehydrated.

In the mice treated with alloxan, the high-frequency conductance of the SC decreased significantly in the first 2 wk after the injection (**Fig 2*A***), but again there was no change in TEWL (**Fig 2*B***). These data definitely indicated that the decrease in the SC water content was not due to the direct toxic effect of streptozotocin or alloxan. They also suggested that the experimentally induced diabetic mice had impaired water homeostasis in the SC without any changes in the barrier function. Moreover, we confirmed a similar decrease in the SC water content in hairless rats (WBN/I 1a-Ht) 4 wk after streptozotocin (40 mg per kg) injection [control, 34.0 ± 10.0 μS (Siemens); streptozotocin, 10.0 ± 1.6 μS (Siemens); $p < 0.05$ ($n = 5$)].

Changes in the amino acid content of the SC To examine the mechanisms underlying the decreased hydration of the SC in the mice with streptozotocin-induced diabetes, we quantified the amino acids in the SC. In this diabetic group, the amino acid content actually showed a slight increase 3 wk after the streptozotocin injection (**Fig 3*A***). According to immunoblotting analysis, the epidermal concentrations of profilaggrin and filaggrin protein, the precursors of the water-soluble amino acids, were almost unaltered in these animals (**Fig 3*B***). These results suggested that the normal metabolism of profilaggrin to amino acids was functional in the epidermis of the streptozotocin-induced diabetic mice.

Changes in the SC lipid content We analyzed the SC lipid contents in the streptozotocin group. Whereas the total SC lipids (per unit area) were higher in the streptozotocin group than in the control group 3 wk after the injection (**Table II**), the SC triglyceride content was significantly decreased after the induction of diabetes. It was interesting to note that the ratios of ceramides and cholesterol to the total lipids remained unchanged after the induction of diabetes, whereas the ratio of triglycerides to the total lipids decreased significantly, and the ratios of cholesterol-wax ester and fatty acids to the total lipids both increased. This change suggested that the triglycerides were being abnormally metabolized in the sebaceous glands of the streptozotocin-induced diabetic mice. Histochemical staining of skin with oil red O, however, showed no special changes in the size or number of sebaceous glands in the streptozotocin group (**Fig 4**).

Changes in the epidermal proliferation and corneocyte surface area At 3 wk after the induction of diabetes, the epidermis of the streptozotocin group was thinner than that of the control group (streptozotocin, 22.2 ± 0.8 μm *vs* control, 28.3 ± 0.9 μm; $n = 5$, $p < 0.005$). The ratio of PCNA-positive basal

Table I. Effects of diabetic induction on plasma glucose content and transaminase activity. Glucose content and transaminase activity were measured at the indicated times after a single injection of diabetes-inducing agent or buffer.

		Control			Treated		
Treatment	Days after the injection	Glucose (mg/dL)	GOT (IU/L)	GPT (IU/L)	Glucose (mg/dL)	GOT (U/L)	GPT (IU/L)
STZ	2 days	213 ± 3.5 (5)	N.D.	N.D.	337 ± 22.6 (10)[a]	N.D.	N.D.
	2 weeks	214 ± 3.9 (5)	N.D.	N.D.	567 ± 54.4 (10)[a]	N.D.	N.D.
	3 weeks	211 ± 4.1 (5)	N.D.	N.D.	682 ± 31.2 (10)[a]	N.D.	N.D.
	4 weeks	221 ± 8.7 (5)	22 ± 1.0 (5)	9.4 ± 0.6 (5)	709 ± 33.4 (10)[a]	24 ± 2.3 (10)[b]	11 ± 1.2 (10)[b]
ALL	2 days	215 ± 3.7 (10)	N.D.	N.D.	689 ± 22.4 (20)[a]	N.D.	N.D.
	2 weeks	218 ± 5.5 (10)	27 ± 1.8 (5)	12 ± 1.1 (5)	730 ± 16.2 (20)[a]	34 ± 3.8 (10)[b]	14 ± 1.7 (10)[b]
	3 weeks	220 ± 3.0 (10)	27 ± 1.4 (5)	10 ± 0.7 (%)	811 ± 16.3 (20)[a]	60 ± 5.9 (10)[b]	18 ± 1.9 (10)[a]

Values represent mean with SEM CONTROL, buffer-injected group; STZ, streptozotocin induction (150 mg per kg); ALL, alloxan induction (45 mg per kg); GOT, glutamic-oxaloacetic transaminase; GPT, glutanic-pyruvic transaminase.

[a]Significant difference between diabetic and control groups ($p < 0.01$).

[b]No significant difference between diabetic and control groups; ND, not determined; the number of animal per group is shown in parentheses.

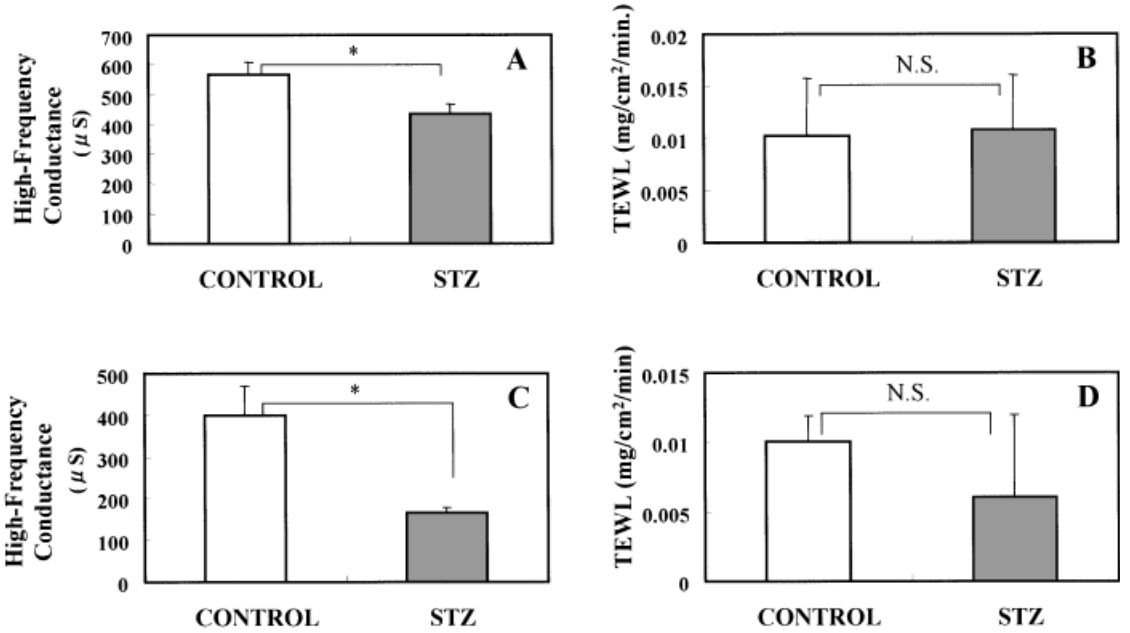

Figure 1. Mice with streptozotocin-induced diabetes show decreased water content of the SC without impairment of the water barrier function. High-frequency conductance (*A,C*) and TEWL (*B,D*) were measured at 2 wk (*A,B*) and 3 wk (*C,D*) after a single injection of streptozotocin (150 mg per kg). CONTROL, buffer-injected group; STZ, streptozotocin group. *Significant ($p < 0.05$); N.S., not significant. Values represent mean with SEM from five animals per group.

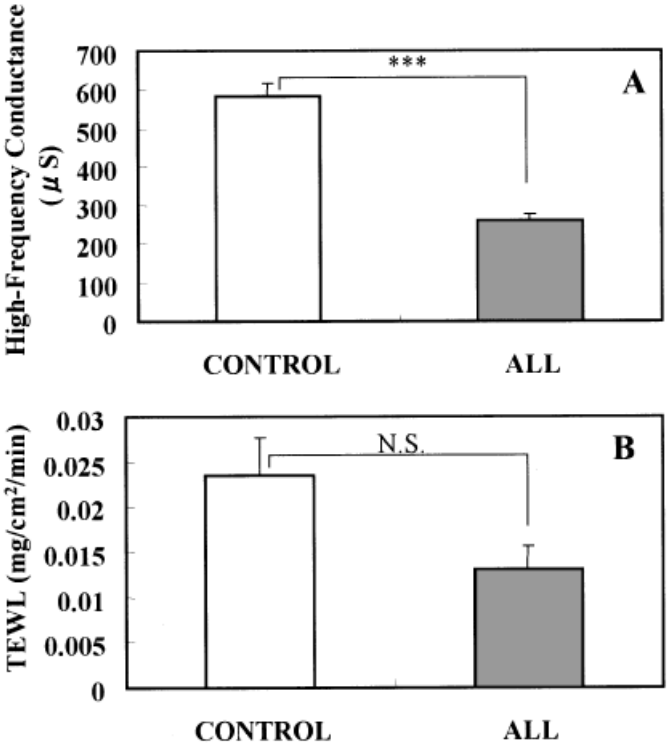

Figure 2. Mice with alloxan-induced diabetes show decreased water content of the SC without impairment of the water barrier function. High-frequency conductance (*A*) and TEWL (*B*) were measured at 2 wk after a single injection of alloxan (45 mg per kg). CONTROL, buffer-injected group; ALL, alloxan group. ***Significant ($p < 0.001$); N.S., not significant. Values represent mean with SEM from five animals per group.

cells to the total basal cells in the epidermis was significantly lower in the streptozotocin group than in the control (**Fig 5**), reflecting the decreased epidermal DNA content in the former (streptozotocin, 23.4 ± 2.1 μg per cm^2 *vs* control, 29.3 ± 0.6 μg per cm^2;

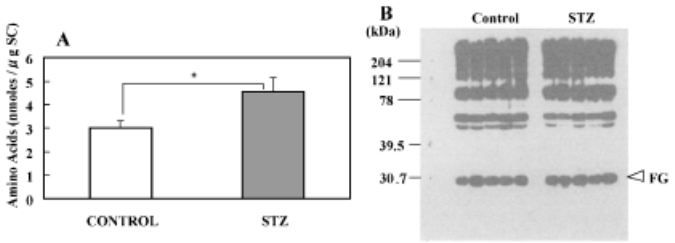

Figure 3. Streptozotocin-induced diabetic mice show normal amino acid production in the SC. The amino acid content in the SC (*A*) and epidermal (pro)filaggrin content (*B*) were assayed at 3 wk after a single injection of streptozotocin (150 mg per kg). CONTROL, buffer-injected group; STZ, streptozotocin group. *Significant ($p < 0.05$). Values represent mean with SEM from five animals per group. FG shows filaggrin monomer protein.

Table II. Streptozotocin-induced diabetes decreased triglyceride content in the SC. The SC lipids were assayed at 3 wk after a single injection of streptozotocin (150 mg per kg). The content was normalized to the surface area

	Content (μg/cm^2) (%)		
Lipids	Control	STZ	
Ceramide I	1.5 ± 0.2 (1.6 ± 0.2)	1.9 ± 0.1^a	(1.6 ± 0.1)^c
Ceramide II-V	9.1 ± 1.1 (10.0 ± 1.6)	15.5 ± 1.0^b	(12.9 ± 1.2)^c
Cholesterol	9.8 ± 1.3 (10.8 ± 1.6)	17.2 ± 1.4^b	(14.1 ± 0.9)^c
Fatty acids	4.5 ± 0.6 (5.0 ± 0.8)	8.3 ± 0.3^b	(6.8 ± 0.3)^a
Triglycerides	20.6 ± 6.9 (24.3 ± 7.5)	4.0 ± 4.1^a	(3.4 ± 3.5)^a
Wax/Choresterol esters	22.6 ± 1.0 (24.5 ± 1.3)	41.0 ± 3.3^b	(33.7 ± 2.1)^b
Total lipids	92.5 ± 3.8	121.6 ± 5.9^b	

CONTROL, buffer-injected group; STZ, streptozotocin group.
[a]Significant ($p < 0.05$),
[b]significant ($p < 0.005$);
[c]not significant.
Values represent mean with SEM from five animals per group.

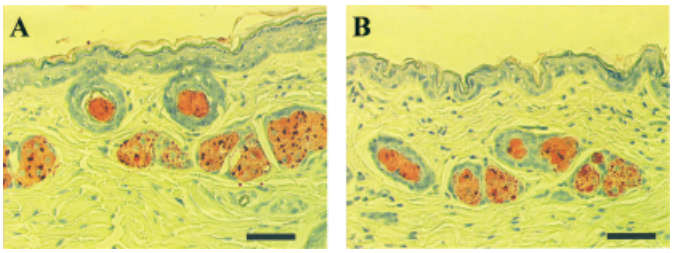

Figure 4. Oil Red O stain of skin of streptozotocin-induced diabetic mice. At 3 wk after the streptozotocin induction, frozen sections of mouse skin were stained using oil red O. (*A*) Buffer-injected group; (*B*) streptozotocin group. *Scale bar* = 50 μm.

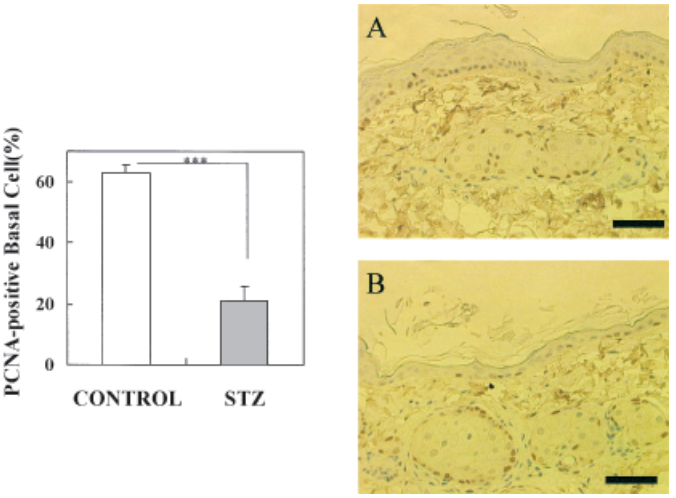

Figure 5. The epidermis of mice with streptozotocin-induced diabetes shows decreased proliferation of basal cells. At 3 wk after a single injection of streptozotocin (150 mg per kg), specimens were fixed and immunostained using anti-PCNA antibody. PCNA-positive basal cells were counted. CONTROL (*A*), buffer-injected group; STZ (*B*), streptozotocin group. ***Significant ($p < 0.001$). Values represent mean with SEM from four animals per group.

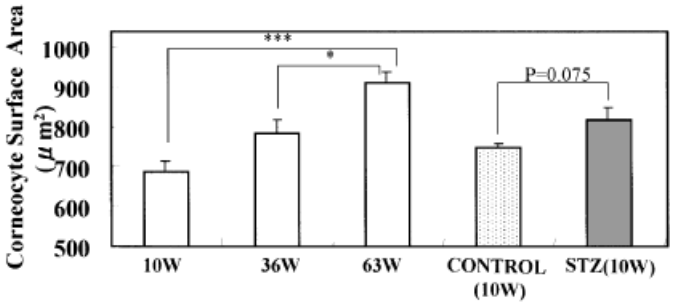

Figure 6. Surface areas of corneocytes in streptozotocin-induced diabetic mouse. The corneocyte surface areas were measured 3 wk after a single injection of streptozotocin (150 mg per kg), and also at indicated ages (10, 36, 63 wk). CONTROL, buffer-injected group; STZ, streptozotocin group. ***Significant ($p < 0.001$); *significant ($p < 0.05$). Values represent mean with SEM from five animals per group.

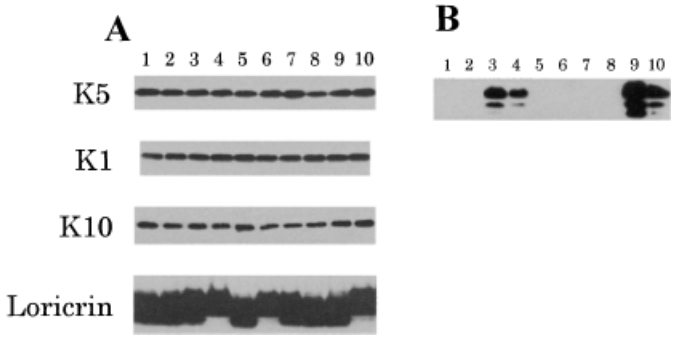

Figure 7. Effects of streptozotocin-induction on the differentiation marker proteins of the epidermis. At 3 wk after the induction, epidermal protein was extracted and applied to western blotting (*A*). Two micrograms [for keratin 1 (K1), keratin 5 (K5), and keratin 10 (K10)] or 20 μg (for loricrin) of epidermal protein was loaded per lane. *Lanes 1–5*, buffer-injected group; *lanes 6–10*, streptozotocin group. At the same time, the SC protein was extracted and applied to western blotting using anti-loricrin antibody (*B*). Twenty micrograms of SC protein was loaded per lane. *Lanes 1 and 2*, buffer-injected group (10 wk); *lanes 3 and 4*, streptozotocin group (10 wk); *lanes 5 and 6*, normal mice (16 wk); *lanes 7 and 8*, normal mice (48 wk); *lanes 9 and 10*, aged mice (80 wk).

$n = 5$, $p < 0.05$). The corneocyte surface area is reported to grow in senile xerosis (Corcuff and Leveque, 1993; Hara *et al*, 1993) and shrink in atopic xerosis (Watanabe *et al*, 1991), reflecting the epidermal turnover rate. The corneocyte surface area in the streptozotocin group tended to increase above the control level (**Fig 6**), with increases in the number of corneocyte layers in the SC (control, 6.3 ± 0.1 μg per cm^2 *vs* streptozotocin, 7.3 ± 0.4; $n = 5$, $p < 0.05$). These results suggested that the epidermal cell turnover in the streptozotocin-induced diabetic mice progressed at a slower rate.

Changes in the epidermal differentiation Prompted by an earlier report demonstrating an insulin-induced stimulation of keratinocyte differentiation *in vitro* (Wertheimer *et al*, 2000), we decided to examine whether streptozotocin could induce any changes in the epidermal differentiation. In a comparison with the control group by western blotting, we found no special changes of the epidermal proteins, keratin 1, keratin 5, keratin 10, and loricrin (58–90 kDa), in the streptozotocin animals (**Fig 7*A***); however, we found that three peptides (34 kDa, 36 kDa, and 43 kDa) observed only in the streptozotocin group (**Fig 7*B***) cross-reacted with anti-loricrin antibody in the SC proteins of the aged mice.

DISCUSSION

In this study, we confirmed for the first time that experimentally induced diabetic hairless mice showed definite functional changes in SC hydration together with alterations in the epidermal proliferation. The decrease in SC water content was not accompanied by impairment in the SC barrier function. The state of SC hydration is known to be regulated by three factors in the SC: the water-soluble natural moisturizing factor (Horii *et al*, 1989), intercellular lipids (Imokawa *et al*, 1991b), and sebum lipids (O'goshi *et al*, 2000). The SC levels of amino acids (Horii *et al*, 1989; Denda *et al*, 1992; Tanaka *et al*, 1998) and ceramides, the main components of intercellular lipids (Imokawa *et al*, 1991a; Akimoto *et al*, 1993; Yoshikawa *et al*, 1994), are reported to decrease in the dry skin observed in the atopic dermatitis and aged skin. Dry skin of senile xerosis is characterized also by significant decreases in triglycerides (Hara *et al*, 1993). In this study, however, we have demonstrated that the decreased water content in the SC of diabetic animals occurs independently of the changes in the substances of the SC, with the exception of the triglycerides. Thus, the decreased water content in the SC is likely to be associated with factors unrelated to the epidermal differentiation. For example, the reduced state of SC hydration may be elicited by the

hyperglycemic condition and/or the inhibition of the insulin signal transduction.

In our experiments, we could not find any impairment of profilaggrin processing or changes in differentiation marker proteins keratin 1, keratin 10, and loricrin in the epidermis of the diabetic animals after streptozotocin treatment.

PKC isoforms are reported to be involved in the differentiation of keratinocytes (Lee *et al*, 1998; Takahashi *et al*, 1998). Keratinocytes contain diacylglycerol-activated PKC such as PKC-α, -δ, -ε, and -η (Reynolds *et al*, 1994; Mitev and Miteva, 1999), and two of these PKC, PKC-α and -η, are known to play an important part in keratinocyte growth and differentiation. Interestingly, hyperglycemia has been reported to induce an increase in the diacylglycerol content in various tissues, such as the retina, aorta, heart, and glomeruli, thereby activating PKC isoforms (Koya and King, 1998; Kang *et al*, 1999). Whereas it remains unknown whether hyperglycemia activates PKC in keratinocytes as it does in other tissues, our present results suggest that PKC is activated under a hyperglycemic condition as well as during normal differentiation. In any case, these points should be investigated further in the future.

The processing of the SC protein may undergo changes in the diabetic state similar to those noted in aged skin. Loricrin, a protein produced in the terminal differentiation process to cross-link immediately the cornified envelope, is very difficult to solubilize (Candi *et al*, 1995). In fact, using the SC protein from normal young mice, the peptides that cross-reacted with anti-loricrin antibody were extremely difficult to solubilize. Using SC proteins from diabetic mice and aged mice, however, we found that the loricrin-derived peptides could be easily solubilized, suggesting that some sort of changes occurred in the maturation, proteolysis, and/or oxidation stages of loricrin processing. This finding might have been due to a decreased turnover rate of the epidermis.

We noted that the streptozotocin-treated mice showed lower proliferative activity of the epidermal basal cells and a reduced epidermal DNA content, together with an increase in the number of corneocyte layers. We also found that the corneocytes were larger in the diabetic animals than in the controls, suggesting that the rates of turnover in both the epidermis and SC were retarded. Similar alterations have been observed in aged human skin (Grove and Kligman, 1983; Hara *et al*, 1993), and enlarged corneocytes have been demonstrated in diabetic patients (Yajima *et al*, 1991). The attenuated insulin signal transduction has been speculated to be important in the growth and differentiation of keratinocytes. Keratinocytes have constituent insulin receptor (Wertheimer *et al*, 2000), and insulin stimulates keratinocyte migration (Benoliel *et al*, 1997) and proliferation (Tsao *et al*, 1982). Recently, Wertheimer *et al* (2000) showed that insulin and insulin-like growth factor also regulated the differentiation of keratinocytes. In experiments using insulin receptor null mice, the same group also reported a reduced proliferation of epidermis similar to that seen in our diabetic mice (Wertheimer *et al*, 2001). On the other hand, another study on diabetic skin has shown a downregulation in the expression of nerve growth factor, an autocrine growth factor of keratinocytes (Anand *et al*, 1996), and an upregulation in the expression of nerve growth factor receptors (Terenghi *et al*, 1997). Thus, abnormal signal transduction of insulin and/or nerve growth factor may also be related to the decreased epidermis turnover in our diabetic mouse model.

It has already been mentioned that the streptozotocin-induced mice exhibited a significant decrease in the SC triglyceride content in parallel with an increase in wax/cholesterol esters. Interestingly, this implies that there may be changes of the lipid metabolism in the sebaceous glands. A similar decrease in the SC triglycerides has been observed in aged human SC (Saint Léger *et al*, 1989; Akimoto *et al*, 1993; Hara *et al*, 1993). Insulin is reported to stimulate the synthesis of fat in the adipocytes (Paulauskis and Sul, 1988; Jensen *et al*, 1989). Sztalryd and Kraemer (1995) reported that hormone-sensitive lipase activity was stimulated in the adipocytes of streptozotocin-induced diabetic rats. The observed decrease of triglycerides in the SC may be due to a similar activation of lipolysis in the sebaceous glands with a consequential change in the sebum composition. A decrease of triglycerides observed in senile xerosis patients (Hara *et al*, 1993) does not rule out the important role of triglycerides in moisturizing the SC. It is well known that lipid extraction of the skin surface with acetone induces a significant reduction in SC hydration, even if continued for only short time. In the seborrheic areas, such as the face and scalp, however, this decreased SC hydration returns to the pretreatment levels within a few hours with a replenishment of skin surface lipids by sebum secretion (Tagami, 1989; O'goshi *et al*, 2000). These reports indicate that sebum plays an important part in the regulation of the hydration state of the skin surface. In contrast, there are some elderly persons without any xerosis change despite the fact that their SC triglyceride content is comparable with those of xerosis patients (Akimoto *et al*, 1993).

Moreover, we cannot exclude the possibility that several heretofore unknown mechanisms unrelated to the effects of amino acids, intercellular lipids, and sebum in the SC have some involvement in the decreased SC water content of this diabetic mouse model. One such mechanism could be the involvement of a natural moisturizing factor. To date, few studies have investigated the function of water-soluble substances other than the SC amino acids in the regulation of the hydration state of the skin surface. Another possible mechanism is the involvement of epidermal water movement. Suzuki *et al* (1994) showed an increase in intracellular hydration in nerve edema in diabetic rats, suggesting abnormal water movement in such a state. A chemiosmotic alteration in the SC and/or keratinocytes beneath the SC layer may impair the SC water homeostasis in diabetic mice. One candidate playing an important part in epidermal water movement is aquaporin, an important water channel of various tissues, such as the kidney, lung, retina, and cornea. Our group recently found that aquaporin isoforms are expressed in the keratinocytes and that aquaporin-3 is induced under hypertonic stress (Sugiyama *et al*, 2001). Ma *et al* (2002) reported reduced SC hydration in the hairless aquaporin-3 null mice. Taken together, these results suggest that the epidermal water movement may be related to the hydrated state of the SC.

It was interesting to note that the SC of the diabetic mice exhibited four features also seen in senile xerosis: reduced SC hydration, impaired turnover of the epidermis, accumulated corneocyte layers, and decreased triglyceride content. Thus, hyperglycemia and/or attenuated insulin signal are thought to enhance the aging process of the skin. Another interesting finding is the discovery of insulin resistance in aging subjects and subjects with Werner's syndrome, which is a form of progeria (Izumino *et al*, 1997; Kausch *et al*, 2000). Further, diabetes-like dermal changes such as collagen glucosylation (Schnider and Kohn, 1980) and elastic fiber abnormalities (Braverman and Keh Yen, 1984) are also described in aged skin, suggesting that an attenuated insulin signal in diabetes may be partially involved in the aging process of the skin. In this study we examine the skin of type 1 diabetes, but it may be interesting to evaluate the phenotype of other diabetic models (i.e., type 2 diabetic models). In future the evaluation of the epidermal function of these models and diabetic patients will reveal the relationship between the epidermal function and hyperglycemia or insulin signal transduction.

In conclusion, this study is the first to describe an impaired water homeostasis in the SC of the experimental diabetic hairless mouse. This model is useful for the study of diabetic dermopathy as well as the mechanisms of SC moisturization.

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