

Suppression of Allergic Inflammatory Response in the Skin of Alloxan-Diabetic Rats: Relationship with Reduced Local Mast Cell Numbers

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Key Words

Diabetes • Skin allergy • Mast cells • Extracellular matrix

Abstract

Background: Diabetic patients are refractory to allergic inflammatory diseases. In this study, the influence of alloxan-induced diabetes on allergic skin inflammation was investigated. **Methods:** Diabetes was induced by intravenous injection of alloxan into male Wistar rats, and the analyses were performed 21 days later. Animals were actively sensitized with a mixture of aluminium hydroxide plus ovalbumin and challenged intradermally with ovalbumin on day 14. **Results:** Diabetic sensitized rats exhibited a less pronounced antigen-induced protein extravasation in the dorsal skin when compared with normal animals. Also, fragments of the dorsal subcutaneous tissue from diabetic sensitized rats showed a reduction in histamine release after stimulation with antigen in vitro when compared with fragments obtained from nondiabetic sensitized rats. Optical microscopy analysis revealed that the dorsal skin of diabetic rats showed a marked reduction in dermis thickness, as compared with that seen in normal animals. A significant decrease in the number of skin mast cells was also noted, a phenomenon that paralleled with the reduction in the expression of ex-

tracellular matrix components laminin, fibronectin and collagen. Administration of insulin into diabetic rats restored basal mast cell numbers as well as the levels of laminin, fibronectin and collagen. **Conclusions:** Our findings show that alloxan diabetes induces downregulation of the skin allergic inflammatory response in rats, and this was correlated with reduction in local mast cell numbers and expression of extracellular matrix components. Lastly, these alterations were reversed with insulin treatment.

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Introduction

Allergic diseases such as bronchial asthma, rhinitis and atopic dermatitis present a high incidence in the population of developed and developing countries [1], though the occurrence of such diseases in patients with diabetes mellitus is lower than predicted [2, 3]. It has been reported that in experimental diabetes caused by alloxan administration, there is a clear downregulation of local allergic inflammatory responses, as ascertained by the low-

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er infiltration of eosinophils both in pleural space [4] and lungs [5]. Also, the lethality associated with anaphylactic shock was markedly reduced in the diabetes condition [6]. According to previous observations, this refractoriness is associated with mast cell depletion and overexpression of endogenous corticosteroids [6, 7].

Mast cells are considered crucial effector cells in anaphylaxis and IgE-associated allergic diseases [8]. In mammals and other vertebrates, mast cells reside in virtually all vascularized tissues and are numerous in anatomical sites that are directly exposed to the external environment, including skin, airways and gastrointestinal tract [9]. This differentiation and maturation in the periphery are tightly regulated by various cytokines, resulting in the generation of heterogeneous populations of mast cells that vary in patterns or expression levels of cell surface proteins and granule constituents, such as histamine, heparin and other proteoglycans, as well as serine proteases [10, 11]. The numbers of mast cells at baseline and during inflammatory or immune responses seem to be regulated by local concentrations of factors that influence their local survival and proliferation, as well as by factors that regulate the tissue homing, survival and maturation of circulating mast cell precursors [12]. Extracellular matrix (ECM) [13] proteins also play an important role in regulating mast cell numbers in the periphery, since the interaction between mast cell and ECM proteins such as fibronectin, laminin and collagens is of crucial importance in the migration and distribution of these cells in tissues [14]. Furthermore, mast cells express integrin-type ECM receptors on the cell surface, which are involved in their activation and increased responsiveness following antigenic stimulation [15].

Thus, considering the refractoriness of diabetic patients to allergic inflammation and the few studies addressing skin diseases and diabetes, we investigated the potential influence of alloxan diabetes on allergen-induced inflammatory response in the rat skin, focusing on mast cell population, as well as selected ECM components.

Material and Methods

Chemicals and Antibodies

Alloxan tetrahydrate, ovalbumin (OVA, grade V), bovine serum albumin, *o*-phthalaldehyde, alumen, Tris (hydroxymethyl aminomethane), safranin, Alcian blue, Evans blue dye, and 3-amino-9-ethylcarbazole were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Insulin NPH was obtained from Biobrás (São Paulo, Brazil). All drugs were prepared immediately before use.

Polyclonal rabbit antisera specific for laminin or fibronectin were purchased from Novotec (St. Martin-La-Garenne, France), whereas the peroxidase-coupled goat anti-rabbit IgG was a Sero-tec (Raleigh, N.C., USA) product. We previously showed that the anti-laminin and anti-fibronectin antibodies recognize the corresponding molecules in rat tissues [16]. Appropriate dilutions of both primary and second antibodies were settled in previous experiments.

Animals, Sensitization Process and Antigenic Challenge

Male Wistar rats (180–200 g) were obtained from the Oswaldo Cruz Foundation animal facility. All procedures involving care and use of laboratory animals in this study were examined and approved by the Animal Ethics Committee of the Oswaldo Cruz Foundation (CEUA-FIOCRUZ, license 00085-02). Rats were sensitized with a subcutaneous injection of a mixture containing 50 µg OVA plus 5 mg of alumen [17] and, 14 days after sensitization, challenged with an intradermal injection of OVA (3 and 12 µg/site) into different regions of shaved dorsal skin. Evans blue dye (25 mg/kg) was injected intravenously 10 min before challenge. Thirty minutes after stimulation, the animals were sacrificed in a CO₂ chamber, and the cutaneous responses were assessed as a function of the area (cm²) and intensity of extravasated dye in skin sites, by analysis of digital scanning images of the lesions, using an image analyzer software (Image-Pro Plus 4). Sensitized rats challenged with sterile saline were used as negative controls. In each experiment, we used 4–10 animals per experimental group (see details in figure captions).

Diabetes Induction and Insulin Treatment

Diabetes was induced by a single injection of alloxan, diluted with sterile saline, into 12-hour-fasted anesthetized rats, 7 days before active sensitization. Blood glycemia was determined by means of a glucose monitor in samples obtained from the tail vein. Evaluation of rats turned diabetic after alloxan administration (40 mg/kg, i.v.) showed an elevation in blood glucose levels (from 90.0 ± 4.0 to 445.0 ± 17.0 mg/dl, mean ± SEM; $p < 0.001$) and a significant decrease in body weight (from 260.1 ± 17.0 to 166.2 ± 10.3 g; $p < 0.01$) and insulin levels (from 58.79 ± 5.29 to 11.99 ± 3.08 IU/ml; $p < 0.001$). Insulin quantification was done as following. Blood was taken by cardiac puncture under light ether anesthesia 21 days after alloxan diabetes induction. After blood coagulation, individual sera were collected and stored at –20°C until use. Serum levels of insulin were evaluated by means of radioimmunoassay (ICN Pharmaceuticals, Costa Mesa, Calif., USA) following the manufacturer's guidelines. Insulin NPH (15 IU/kg) was administered subcutaneously 3 days after diabetes induction, once a day during 18 consecutive days. In control animals, the drug was replaced by its vehicle.

Anaphylactic Histamine Release from Rat Subcutaneous Tissue Fragments in vitro

Dorsal skin tissue (hypodermal layer) was removed from actively diabetic or nondiabetic sensitized rats, washed with Tyrode solution and placed in 24-well plates containing HBSS with Ca²⁺ and Mg²⁺. The tissues were challenged with OVA (400 µg/ml), and 30 min later, the plates were centrifuged at 150 g for 10 min, and the samples were collected and added to 0.8 N perchloric acid solution. After centrifugation at 170 g for 10 min, the supernatant was collected and stored at –20°C for histamine quantification as

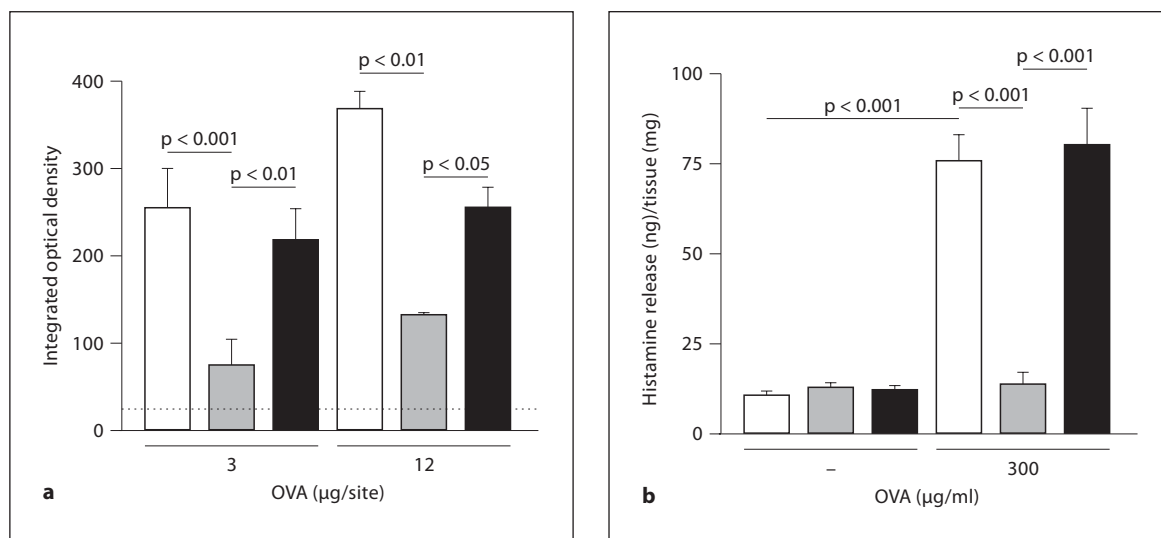


Fig. 1. Decrease in protein extravasation in sensitized diabetic rats and restoring effect of insulin. **a** Levels of protein extravasation in the dorsal skin of normal sensitized (white columns), diabetic sensitized (grey columns) and insulin-treated diabetic sensitized rats (black columns), 1 h after stimulation with OVA (3 and 12 µg/site), as revealed by means of Evans blue extravasation. The dotted line indicates mean values of nonsensitized rats stimulated

with OVA. **b** Effect of alloxan-induced diabetes on OVA-induced histamine release from dorsal skin fragments in vitro. Tissue incubated with medium was used as negative control. Insulin treatment reversed the decrease in protein extravasation and histamine release on diabetes-induced skin refractoriness to antigen challenge. Values represent means $\pm$ SEM from at least 5 animals per group.

previously described [18]. Briefly, this method consists of sample dilution in 0.1 N HCl followed by 0.8 N of NaOH and further addition of the substrate *o*-phthalaldehyde. After 4 min incubation, the reaction was stopped with 3 N HCl and the intensity of fluorescence quantified in a Shimadzu RF1501 spectrofluorophotometer (Japan; excitation at 360 nm, emission at 450 nm). Tissue fragments were dried in incubators at 40°C for 15 min and further weighed. The results were expressed as the amount of histamine release (ng)/mg of tissue.

Histochemical Analysis

Rats were sacrificed in a CO₂ atmosphere, and fragments from hairless skin were removed and fixed in Carnoy's solution for 4 h at room temperature. The specimens were dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin wax, and 5-µm-thick sections were obtained. Samples were then stained with Alcian blue (0.5%, pH = 2.0) and safranin (0.1%) for the identification of mast cells, and the results were expressed as number of cells/µm². Dorsal skin thickness was measured as the distance between the epidermis and dermal-subcutaneous junction. The measurement was done in samples obtained from different sites along the dorsal region of each animal, and the mean values were calculated. The skin thickness from each sample was expressed per µm².

In another set of experiments, Gomori's trichrome dye was used in order to stain collagen fibers. Analyses were performed by optical microscopy (microscope model Olympus BX50), which was coupled to a video camera (Optronics Engineering, DEI-750).

The camera output was processed and analyzed by the image analyzer software, Image-Pro Plus 4. Levels of ECM components were quantified by densitometric scanning (pixels)/µm².

Immunohistochemical Analysis

Sections were cleared in xylene for 5 min, rehydrated by means of a decreasing series of ethanol and washed with distilled water and TBS for 10 min. After delineation with Dako pen, the specimens were incubated with 3% H₂O₂ for 15 min to inactivate the endogenous peroxidase activity. Then, they were incubated with Tris-HCl-2% bovine serum albumin for 1 h at room temperature to avoid nonspecific background staining. Samples were then treated for 12 h at 4°C with anti-laminin or anti-fibronectin antibodies. After rinsing with TBS, the sections were treated with peroxidase-coupled secondary antibody for 1 h at room temperature. Enzyme activity was revealed with the 3-amino-9-ethylcarbazole substrate. Sections were then counterstained with Harris hematoxylin, rinsed in tap water, mounted in an aqueous medium and visualized by optical microscopy; images were ultimately analyzed by an image analyzer software (Image-Pro Plus 4). In control experiments, no immunostaining was observed when primary antibody was omitted.

Statistical Analysis

The data were reported as means $\pm$ standard error and statistically analyzed by ANOVA followed by Newman-Keuls-Student's t test. Probability values (p) of 0.05 or less were considered significant.

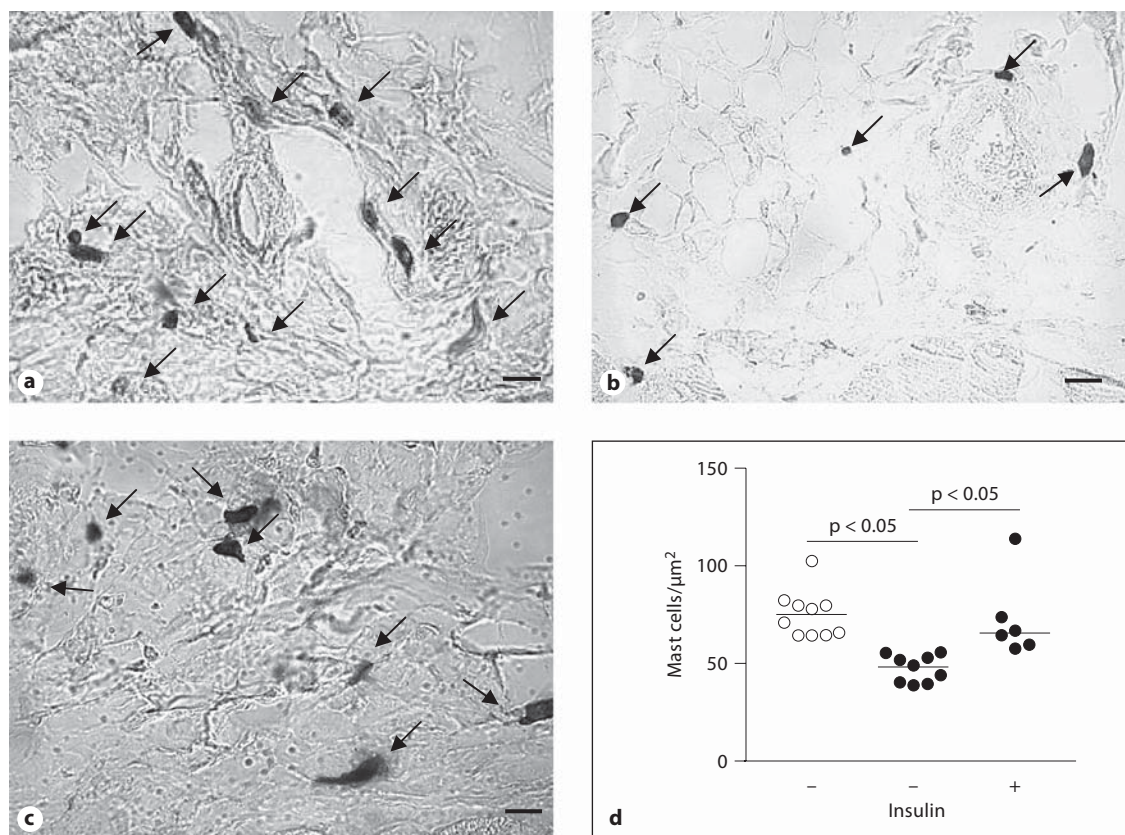


Fig. 2. Decrease in mast cell numbers in the skin of alloxan-induced diabetic rats and restoring effect of insulin. **a–c** Photomicrographs of Alcian blue-safranin-stained mast cells (arrows) in the dermal layer from the dorsal skin from normal (**a**), alloxan-diabetic (**b**) and insulin-treated diabetic (**c**) rats. Bar = 0.1 μm . **d** Numbers of mast cells are significantly reduced in the skin of diabetic rats, as compared with controls, and such a decrease can

be restored by insulin treatment. Individual data recorded in the skin of sensitized (white circles) or diabetic sensitized (black circles) rats, either in the absence or presence of insulin treatment. Horizontal bars represent the mean value for each experimental group. Insulin (15 IU/kg) was administered subcutaneously 3 days after diabetes induction, once a day during 18 consecutive days.

Results

Skin Reactivity following Allergen Stimulation Is Decreased in Alloxan-Induced Diabetic Rats

To evaluate the skin reactivity to allergen challenge, actively sensitized rats were injected intradermally with OVA (3 and 12 $\mu\text{g}/\text{site}$), and the protein extravasation was evaluated. As expected, sensitized rats stimulated with antigen exhibited a dose-dependent increase in the levels of extravasated protein, 1 h after challenge. Nevertheless, under conditions of alloxan diabetes, a significant decrease in the protein leakage was noted with both doses of antigen tested. Diabetic sensitized rats treated with insulin (15 IU/kg, s.c.), once a day for 18 days, clearly reversed the decrease in the allergen-induced skin protein extravasation (fig. 1a).

In another set of experiments, we showed that stimulation with antigen (OVA 300 $\mu\text{g}/\text{ml}$) in vitro caused a marked increase in the amount of histamine released from dorsal skin fragments from sensitized rats, whereas a drastic reduction in the anaphylactic secretion of histamine was observed in the case of tissue from diabetic sensitized rats. No difference in the basal histamine release from the skin was noted under diabetic conditions as compared with controls. Values of tissue histamine release were restored to control in the case of tissue from diabetic sensitized rats treated with insulin (fig. 1b).

Alloxan-Induced Diabetes Decreases the Number of Skin Mast Cells

To evaluate the potential effect of diabetes on the skin mast cell population, tissue fragments were fixed and stained with Alcian blue and safranin dyes, and the number of total mast cells was enumerated as cells per $1 \mu\text{m}^2$. We noted that under normal conditions, mast cells exhibiting connective tissue phenotype (safranin-positive cells) were mainly located in the hypodermis of the dorsal skin of rats (fig. 2a). In diabetic rats, mast cells were detected in a similar distribution pattern, though in significantly lower numbers as compared with those of normal animals (fig. 2b). Treatment with insulin significantly reversed the drop of mast cell numbers in diabetic rats (fig. 2c), under conditions where no alteration in the mast cell population of normal animals was noted (data not shown).

Alloxan-Induced Diabetes Decreases the ECM Layer of the Dorsal Skin

The effect of alloxan diabetes on skin morphology is shown in figure 3. In normal rats, we observed a significant reduction in the dorsal skin thickness along the back, from the proximal to distal regions (fig. 3a). Diabetic rats showed a similar morphological pattern along the dorsal region, but also exhibited a remarkable reduction in skin thickness in the 3 regions analyzed (fig. 3a). As compared with the normal skin pattern in the controls (fig. 3b), we clearly noted that the reduction in skin thickness in diabetic rats occurred more particularly in the space between epidermal layer and smooth muscle cells (dermal layer) (fig. 3c).

Immunohistochemical staining for ECM proteins revealed a strong labeling for laminin and fibronectin in the skin of normal rats, mainly localized in the dermal layer (fig. 4a, b). In diabetic rats, a marked decrease in the deposition of laminin (fig. 4d) and fibronectin

(fig. 4e) was detected in the skin of diabetic rats compared with controls. We also examined the pattern of collagen deposition. Similarly to laminin and fibronectin, the intensity collagen fibers revealed with Gomori's trichrome staining was remarkably reduced in diabetic

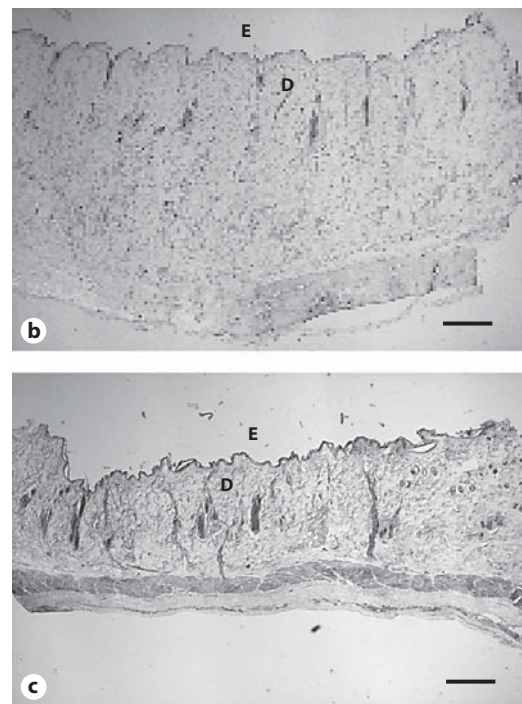
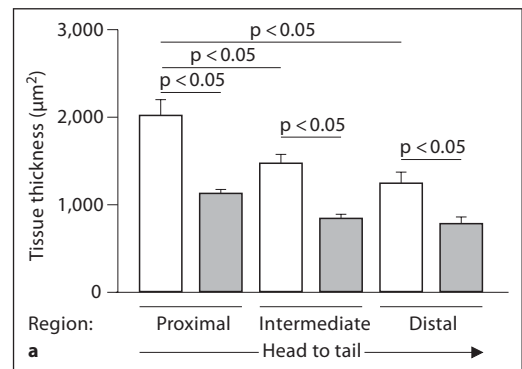
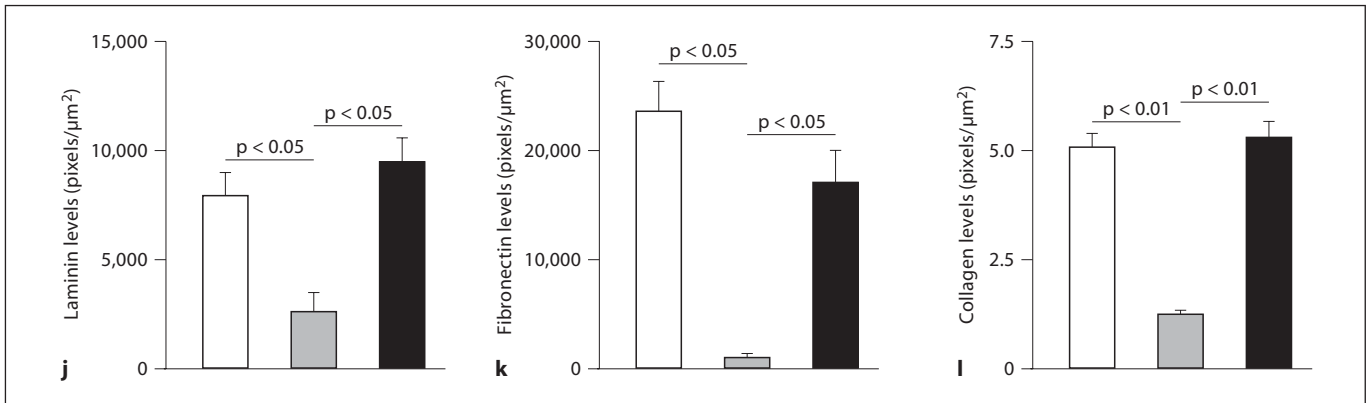
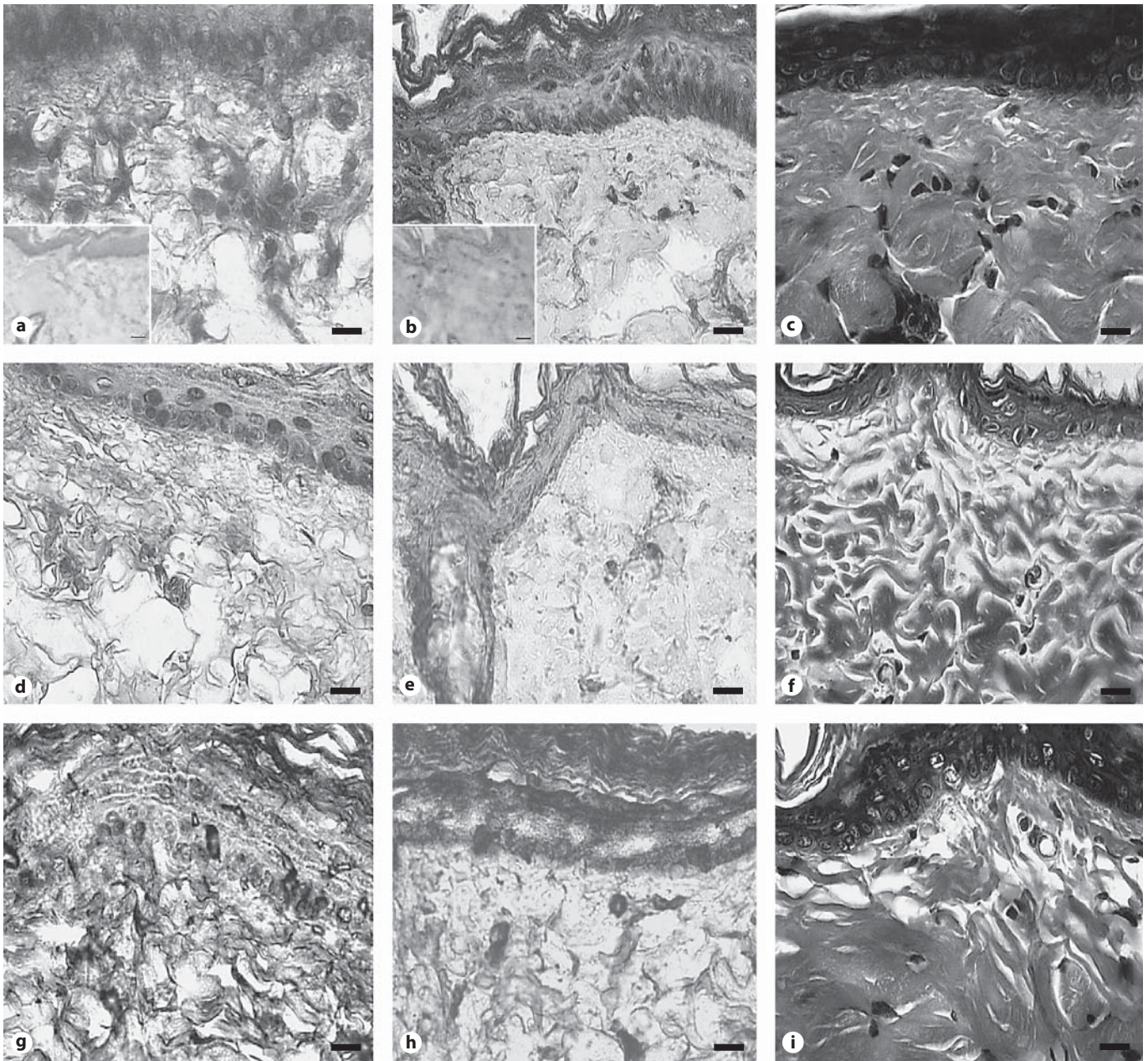


Fig. 3. Decrease in skin thickness in diabetic rats. **a** Quantification of the reduction in skin thickness, in proximal, intermediate and distal segments of the dorsal skin, seen in alloxan-diabetic (grey columns) rats, as compared with controls (white columns). **b, c** Representative photomicrographs of the proximal region of dorsal skin from normal and diabetic rats, respectively. Data are expressed as means $\pm$ SEM of at least 4 animals. Bar = $1.5 \mu\text{m}$. E = Epidermal layer; D = dermal layer of the skin.

Fig. 4. Decrease in the deposition of ECM components in the skin of diabetic rats. Photomicrographs of immunostaining of laminin (**a, d, g**) and fibronectin (**b, e, h**) of dorsal skin sections from rats.

Inserts represent negative controls in which primary antibody was omitted. Sections were counterstained with Harris hematoxylin. Photomicrographs of Gomori's trichrome-stained skin sections (**c, f, i**) depict collagen fibers. Samples were analyzed in normal rats (**a–c**), diabetic rats (**d–f**) and insulin-treated diabetic rats (**g–i**). Bar = $0.1 \mu\text{m}$. Quantitative evaluations of laminin, fibronectin and collagen labeling are seen in panels **j–l**, respectively. As compared with controls (white columns), in all cases, there is a decrease in ECM contents in the skin of diabetic rats (grey columns), and such a decrease is significantly reverted by insulin treatment (black columns).



rats when compared with normal animals (fig. 4f, c). Importantly, we showed that treatment with insulin clearly restored the normal patterns of laminin (fig. 4g), fibronectin (fig. 4h) and collagen deposition (fig. 4i) in the skin of diabetic rats. Quantitative evaluations of laminin, fibronectin and collagen labeling are seen in figure 4j–l.

Discussion

Previous studies described that allergic diseases including asthma and atopic dermatitis occurred less often than expected in patients with diabetes [3]. We previously demonstrated that the population of free mast cells was downregulated in the celomatic cavities of alloxan-diabetic animals, an event that may be at least partially responsible for their refractoriness to antigen challenge [4, 19, 20]. Here, we showed a significant reduction in antigen-evoked skin plasma leakage and histamine release from the skin of diabetic sensitized rats, in good correlation with a decrease in the number of mast cells, as well as in the deposition of ECM proteins such as laminin, fibronectin and collagen. Treatment with insulin restored the protein leakage induced by antigen, mast cell numbers and levels of ECM proteins in the skin of diabetic rats. Altogether, our findings indicate that the refractoriness of diabetic animals to skin allergen challenge is associated with reduction in the mast cell numbers and ECM protein expression, in a clear dependence on the downregulation of endogenous insulin.

It is demonstrated that there is a rapid release of inflammatory mediators after antigen stimulation and that mast cells play a central role in such responses [21]. In the current study, we showed that sensitized rats exhibited an acute and dose-dependent protein extravasation in the dorsal skin, 1 h after OVA provocation. Nevertheless, in the case of diabetic sensitized rats, such a protein leakage was significantly less intense. This phenomenon could be associated with functional changes in the behavior of microvessels and/or decreased responsiveness of endothelial cells to vasoactive agents [22]. Yet, one could argue that the potential contribution of decreased levels of circulating proteins to the lower exudatory response is secondary to an accelerated protein catabolism associated with the diabetic state. This possibility was ruled out since no difference in the serum albumin contents was detected in diabetic rats (1.13 ± 0.8 g/dl) when compared with those from normal animals (1.44 ± 0.8 g/dl). Alternatively, the refractoriness

of diabetic sensitized rats to allergen challenge could be due to a defective response of the IgE mast cell system. We and others reported that augmentation in the total and OVA-specific serum IgE levels, seen after active sensitization, is completely suppressed in diabetic rats [6]. For better evaluation of the contribution of mast cells to the refractoriness to allergen provocation, we investigated the influence of alloxan diabetes on the antigen-provoked histamine release from isolated skin tissue in vitro. Although dorsal skin fragments from sensitized rats were quite sensitive to in vitro OVA provocation, the tissue recovered from diabetic sensitized rats showed refractoriness to antigen challenge.

We further approached the influence of the diabetic state upon the local mast cell population and noted that those mast cells located in the skin (particularly within the hypodermis layer) are also negatively regulated under diabetic conditions. Similar findings were noted in case of free mast cells in the pleural and peritoneal cavities of diabetic rats, a phenomenon directly correlated with diminished antigen-provoked protein leakage and leukocyte infiltration [4, 18]. Under conditions of reconstitution of the pleural mast cell population in diabetic rats, by means of an adoptive transfer system, restoration of the allergic inflammatory process [18] was seen, reinforcing the proposition that downregulation of mast cells is at the origin (at least partially) of the refractoriness of diabetic animals to antigen.

We noticed that the dorsal skin of alloxan-diabetic rats also underwent structural changes as ascertained by a significant reduction in the thickness all along the whole back. A marked decrease in the deposition of ECM proteins, including collagen, laminin and fibronectin, was also detected. The localization of mast cells in tissues is dependent on their interaction with ECM proteins, which are thus considered to play a crucial role for the homing, maturation, function and survival of these cells [13]. Mast cell numbers and reactivity were shown to be downregulated under diabetic conditions, in a clear association with increased levels of apoptosis [23]. Thus, we can speculate that changes in the ECM protein contents could contribute to the mast cell depletion seen in diabetic rats. One plausible explanation for the decrease in ECM components is the occurrence of chronic hyperglycemia associated with diabetes in general. Consistent with this idea, it was reported that glucose has the ability to reduce collagen biosynthesis and collagen crosslinking [24], which might explain the decreased amounts of interstitial collagen and possibly the poor wound healing associated with diabetes [25].

In the present study, we showed that insulin treatment in diabetic animals restored basal levels of mast cells as well as of ECM proteins, indicating that the lack of this hormone seems to contribute to the skin structural changes in diabetes. The activity of insulin to induce cell proliferation and activation was verified in different biological systems [26]. Thus, one possibility is that insulin might be acting in the control of proliferation and/or survival of mast cells and fibroblasts as well as of ECM protein production in order to maintain the skin structure [27, 28]. Evidence has shown that low levels of insulin were clearly associated with hypercorticism in diabetes and that treatment with insulin impairs the increase in the glucocorticoid levels [7, 29]. Glucocorticoids have been directly implicated in the deficit noted in collagen deposition and differentiation of ECM under conditions of diabetes [25]. Thus, it is reasonable to speculate that the glucocorticoids can also be implicated in the abnormalities noted in the skin of alloxan-diabetic rats. Experiments are now underway to clarify this point.

In conclusion, our findings indicate that alloxan diabetes induces refractoriness to antigen provocation in the dorsal skin of rats, in parallel with depletion of local mast cell population and ECM components including laminin fibronectin and collagen. Treatment with insulin restored basal mast cell population and ECM protein expression, reinforcing the idea that abnormalities in the skin of alloxan-diabetic rats may be a consequence of insulin deficiency.

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