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ORIGINAL PAPER

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Pretreatment with topical all-*trans*-retinoic acid is beneficial for wound healing in genetically diabetic mice

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Abstract ***Objective:*** **Topical pretreatment with all-*trans*-retinoic acid (atRA) is known to improve healing of cutaneous wounds. We tested the effect of atRA on wound healing of genetically diabetic db/db mice. It is known that cutaneous wounds of db/db mice show delayed wound healing due to impaired wound contraction, delayed granulation tissue formation and underexpression of keratinocyte growth factor (KGF).** ***Methods:*** **0.1% atRA in 100 mg aqueous gel was applied to the back skin of db/db mice as well as to their normal heterozygous littermates, db/+ mice, for five consecutive days, and 2 days after completion of the atRA treatment, two round excisional wounds were created down the panniculus carnosus with a 6-mm punch biopsy on the back skin of each mouse.** ***Results:*** **After 5 days treatment with 0.1% atRA, significant hypertrophy of the epidermis and dermis, neovascularization, and inflammatory cell invasion were seen in the skin of the db/db mice, but these effects were seen only weakly in db/+ mice. Wounds in atRA-treated db/db mice closed more rapidly than those in vehicle-treated db/db mice. KGF mRNA expression, which is usually significantly lower in db/db mice than in normal mice, in wounds of atRA-treated db/db mice on day 1 of treatment was as strong as in db/+ mice.** ***Conclusion:*** **Pretreatment with atRA reversed the impaired wound healing in db/db mice.**

Keywords All-trans retinoic acid · Diabetic mouse · Wound healing

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Introduction

Retinoic acid (RA) improves the clinical symptoms of acne vulgaris, psoriasis and skin photoaging [1]. Histologically, topical treatment with RA induces hypertrophy in the epidermis and dermis via an increase in dermal vasculature and invasion of inflammatory cells. RA has several contradictory effects on wound healing. For example, systemic application of RA has deleterious effect on healing of full-thickness skin wounds and corneal wounds of diabetic animals [2, 3]. On the other hand, systemic RA reverses delayed wound healing in steroid-treated animals [4–7]. RA is also known to improve wound healing of abraded skin by acceleration of epidermal turnover when it is applied topically prior to wounding [8]. Topical pretreatment with RA also enhances healing of full-thickness excisional wounds of photoaged and normal skin [9, 10]. These results suggest that wound healing may be affected by the route or schedule of retinoid administration, and that, at least in some skin conditions, topical pretreatment may be the most effective method of administration of all-*trans*-RA (atRA).

We hypothesized that impaired wound healing of genetically diabetic db/db mice may be reversed by topical pretreatment with atRA. Db/db mice show clinical symptoms similar to insulin-resistant diabetes mellitus patients, such as severe obesity, hyperglycemia, hyperinsulinemia, and insulin resistance [11–14]. These symptoms were derived from genomic mutation in the leptin receptor. Db/db mice are also known to show impaired cutaneous wound healing, as is universally observed in diabetic patients [11]. Wound healing of db/db mice is characterized by weak contraction, slow inflammatory cell invasion and delayed granulation formation. The characteristic anatomy of the skin of db/db mice, including thick subdermal fat and an extremely thin dermis, may also contribute to impairment of wound healing.

Many growth factors and their receptors, such as FGF, FGF receptors, KGF, IGF and VEGF, have been found to be aberrantly expressed in wounds of db/db mice [11, 12]. Of these, the factor that is considered to be the most important for delayed wound healing in db/db mice is KGF

[15]. In normal mice, the mRNA levels of KGF are acutely upregulated early in wound healing [16, 17]. However, KGF mRNA levels in db/db mice are unchanged throughout the healing process [15]. Since upregulation of KGF mRNA is one of the earliest events of wound healing, KGF is considered to be an important factor that might regulate downstream events during wound healing.

Materials and methods

Animals

Female C57BLK/ksj db/db mice at 8–12 weeks of age (Saitama Experimental Animals Supply Company, Saitama, Japan) and their normal littermates (db/+ mice) were used throughout this study. Animal experiments were performed according to the Laboratory Animal Guidelines of Tokyo University. All animal procedures were performed under general anesthesia with diethyl ether. Three independent experiments were performed, and 12 to 15 mice were used in each experiment. The back skin of each mouse was shaved and topically treated for five consecutive days with 100 mg 0.1% atRA (Sigma Aldrich, Tokyo, Japan) in aqueous gel vehicle or 100 mg aqueous gel without atRA. Scaling was graded by three investigators according to the visual scoring method of Effendy et al. [18]: 0, no scaling; 1, weak scaling; 2, moderate scaling; 3, large flakes, intense peeling.

On the 2nd day after completion of atRA treatment, two 6-mm round excisional wounds, down the panniculus carnosus and its associated fascia, were created in each mouse. The wound area was calculated as the product of the long and short diameters of each wound. Mice were killed on days 1, 3, 7 and 14 by cervical dislocation under general anesthesia with diethyl ether. Wound tissue was processed for histology and RT-PCR.

Histology

The mice were injected subcutaneously with 30 μg/g 5-bromo-2′-deoxyuridine (BrdU labeling reagent; Amersham Pharmacia, Shinjuku, Tokyo) 3 and 24 h before they were killed. Skin samples were fixed in 10% formalin, dehydrated and embedded in paraffin. Paraffin sections were cut at a thickness of 5 μm. Sections were rehydrated and stained with hematoxylin and eosin, and with Gomori's trichrome stain. Immunohistochemistry to determine the level of BrdU incorporation into cells was performed as follows. Sections were incubated in 4 *N* HCl for 30 min at 37 °C followed by 0.1 *M* borax buffer, pH 9.0, for 10 min at room temperature. The specimens were then treated with anti-BrdU monoclonal antibody (Amersham Pharmacia) followed by biotinylated anti-mouse IgG. The specimens were then incubated in ABC reagent (Vector, Burlingame, Calif.), and developed with 3,3′-diaminobenzidine.

RT-PCR

mRNA was extracted from a 2-mm wound margin using ISOGEN LS (Nippon Gene, Shinjuku, Tokyo). The mRNA (1 μg in a total volume of 20 μl) was transcribed to cDNA by AMV reverse transcriptase (Promega Japan, Higashinihonnbashi, Tokyo) at 37 °C for 1 h. For semiquantitative detection of KGF and β-actin cDNA, 1 μl cDNA in a total volume of 50 μl (50 m*M* KCl, 15 m*M* Tris-HCl, pH 8.0, 4 m*M* $MgCl_2$) was amplified with Ampli-Taq Gold (PE Biosystems, Roppongi, Tokyo) in a PC-960G thermal cycler (Cosmo Bio, Toyo, Tokyo). The sequences of the oligonucleotide primers used for amplification of KGF were TTGCAATGAACAAGGAAGGA and GAATTCTATCTTGCAATGAA, and those for β-actin were TGAGGAGCACCCTATGCTGC and TAGCCCTCGTAGATGGG. Amplification was carried out as follows: pre-PCR step of 10 min at 94 °C; 32 PCR cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C; and a final step of 10 min at 72 °C. We had already confirmed in a preliminary study that the PCR products would be within the linear log phase under these conditions.

Results

The effects of topical application of atRA

Scaling was more intense in db/db mice than in db/+ mice following treatment with 0.1% atRA (Fig. 1). Although

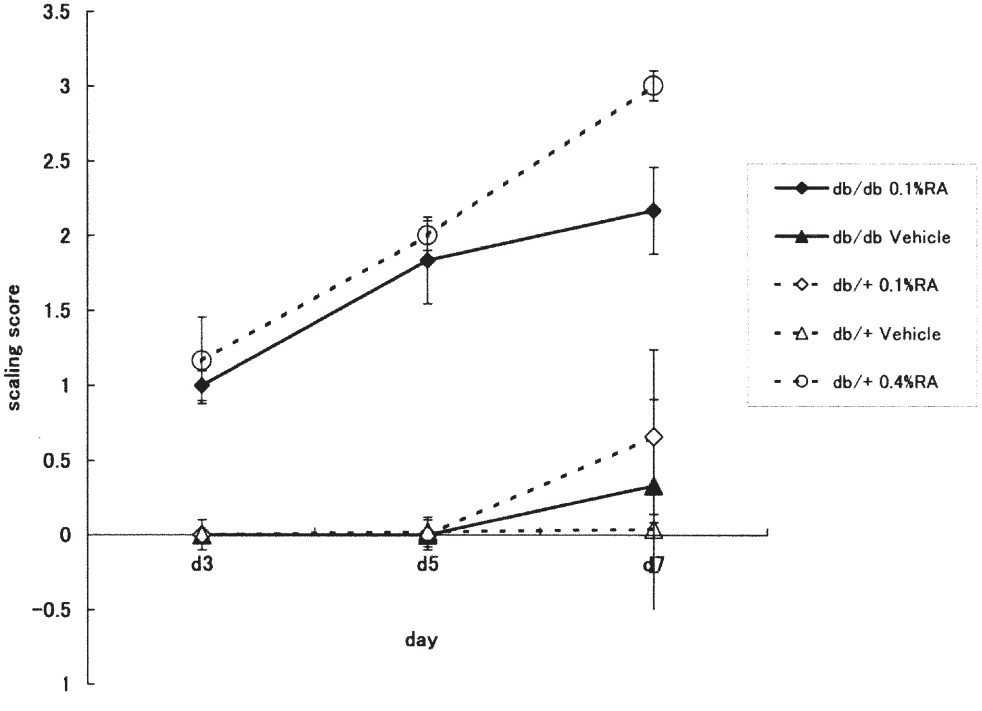

Fig. 1 Scaling score of db/db mice treated with 0.1% atRA, and of db/+ mice treated with 0.1% or 0.4% atRA. Three mice in each group were evaluated. The values are means ± SD of duplicate measurements from three independent experiments

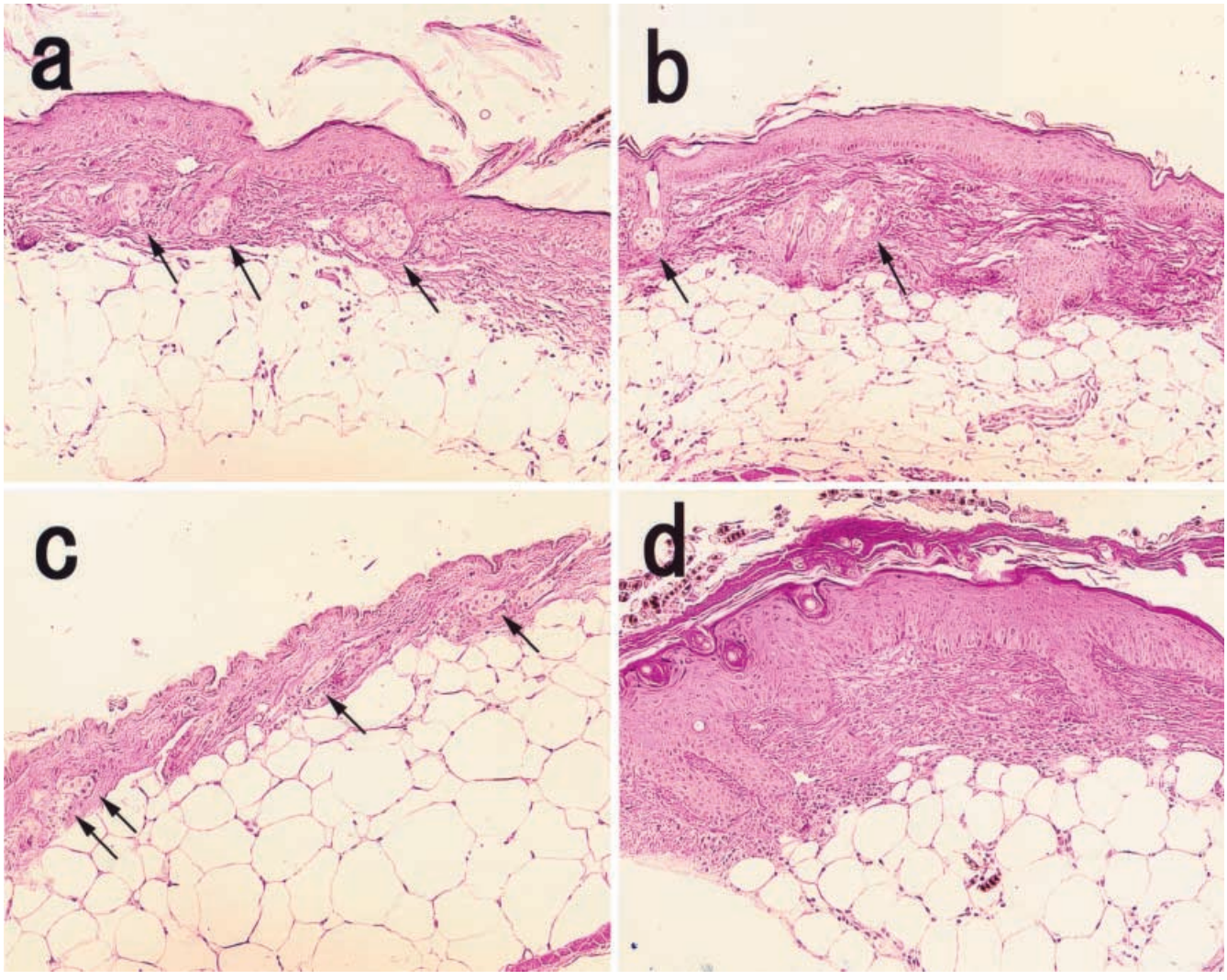

Fig. 2 a–d H&E staining of the back skin of db/+ mice (**a** vehicle-treated mouse, **b** 0.1% atRA-treated mouse) and db/db mice (**c** vehicle-treated mouse, **d** 0.1% atRA-treated mouse). *Arrows* indicate sebaceous glands

0.1% atRA did not induce significant scaling in db/+ mice, higher concentrations of atRA did induce significant scaling in these mice, so the effect of atRA appears to be dose-dependent.

Histologically, the skin of db/+ mouse showed only slight dermal and epidermal hypertrophy after treatment with 0.1% atRA. On the other hand, the skin of atRA-treated db/db mice showed significant hypertrophy of epidermis and dermis, scaling of stratum corneum, an increase in cellularity and proliferation of subepidermal vessels (Fig. 2). Elongation of the rete ridges and atrophy of the sebaceous glands were also characteristic of atRA-treated db/db mice. In vehicle-treated db/db mice, BrdU incorporation was observed throughout the basal layer of the epidermis. However, in atRA-treated db/db mice, BrdU incorporation was greater in the follicular epidermis, but not in the interfollicular epidermis (Fig. 3).

Wound healing of atRA- and vehicle-treated db/db mice

Day 0 after wounding

The wounds in vehicle-treated db/db mice were 20–30% larger than the original wounds, but the wounds in atRA-treated db/db mice did not show enlargement just after wounding (Fig. 4).

Day 1 after wounding

Vehicle-treated db/db mice on day 1 after wounding showed only a small number of inflammatory cells in the wound cleft. Only a small amount of extracellular matrix had formed at this time. However, atRA-treated db/db mice on day 1 showed massive invasion of inflammatory cells in the wound cleft. In addition, a large amount of extracellular matrix staining bluish with trichrome reagent had formed within the granulation tissue, which connected the granulation tissue with the wound margin (Fig. 5a, b).

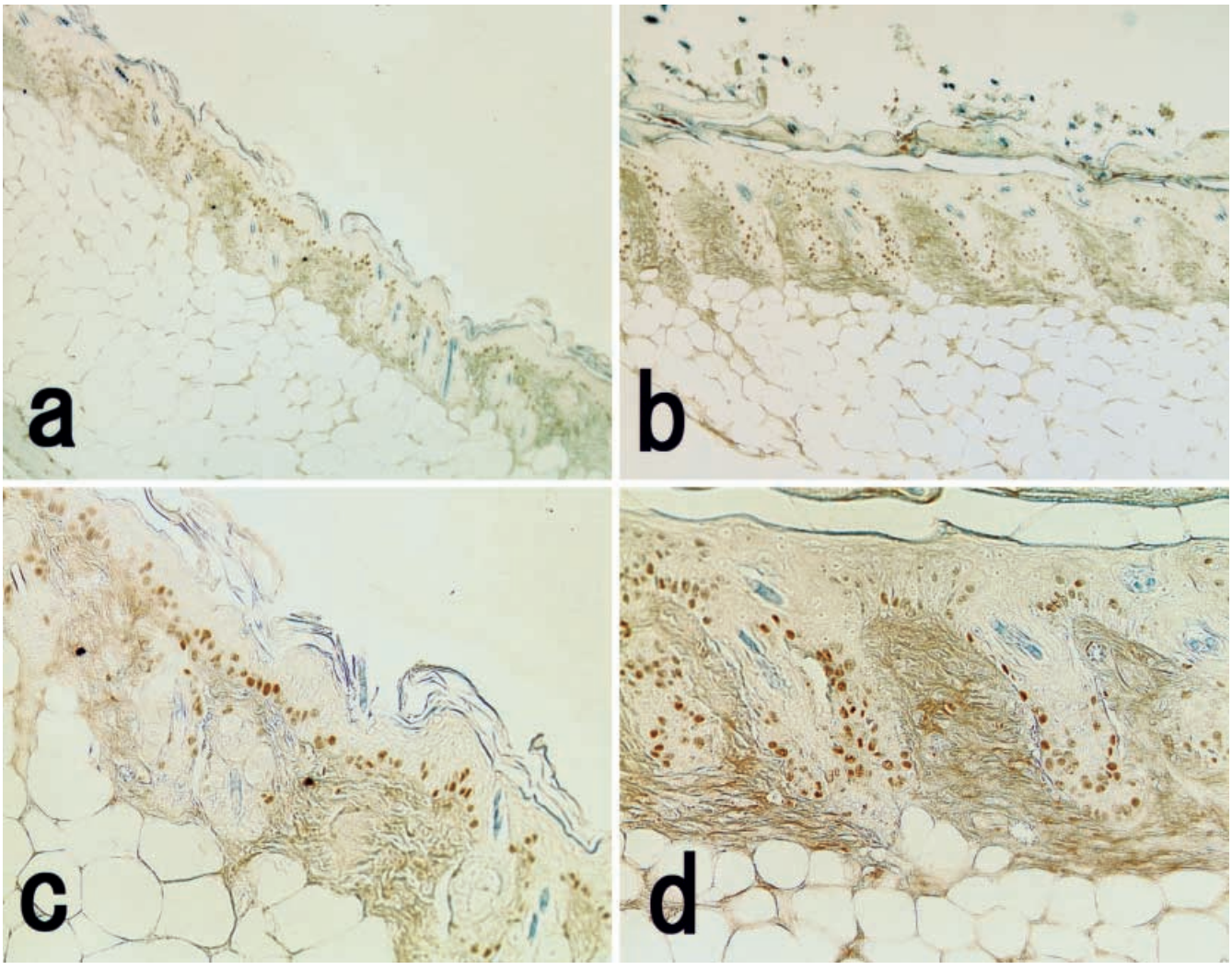

Fig. 3 a–d BrdU staining of back skin of db/db mice treated with either of vehicle (**a**) or 0.1% atRA (**b**) for 5 days. **c**, **d** High-power magnification of **a** and **b** (**a**, **b** ×100; **c**, **d** ×200)

Day 14 after wounding

Macroscopically, 78% of wounds in vehicle-treated db/db mice had not closed on day 14. Histologically there was a large amount of granulation tissue within the wide wound cleft. Epidermal regeneration had started, but due to the distance between the wound margins, there still was an uncovered area within the cleft. On the other hand, 89% of the wounds in atRA-treated db/db mice had already closed by this time. Histologically the wound margins had come close to each other, and reepithelialization was almost complete (Fig. 5c, d).

Expression of KGF mRNA

mRNA expression of KGF at the time of wounding did not show significant differences between atRA- and vehicle-treated db/db mice (data not shown). However, KGF mRNA in the wounds of atRA-treated db/db mice on day 1 after wounding was significantly higher than in vehicle- or sodium lauryl sulfate-treated db/db mice (Fig. 6).

Discussion

In the study reported here we demonstrated that (1) topical treatment with atRA had stronger effects in db/db mice than in db/+ mice, and that (2) atRA pretreatment reversed delayed wound healing in db/db mice.

It is noteworthy that atRA induced cutaneous changes in db/db mice much more strongly than in db/+ mice. This does not mean that db/+ mice are totally refractory to the effects of atRA, but that db/db mice are more sensitive to atRA than db/+ mice, because the latter showed effects similar to those seen in db/db mice when they were treated with higher concentrations of atRA. It is not clear whether this increased sensitivity was due to downstream events resulting from the leptin receptor deficiency, or due to loss of barrier function. Similar model-specific sensitivity has been observed in other experiments. For example, atRA increases the synthesis of collagen and hyaluronic acid in

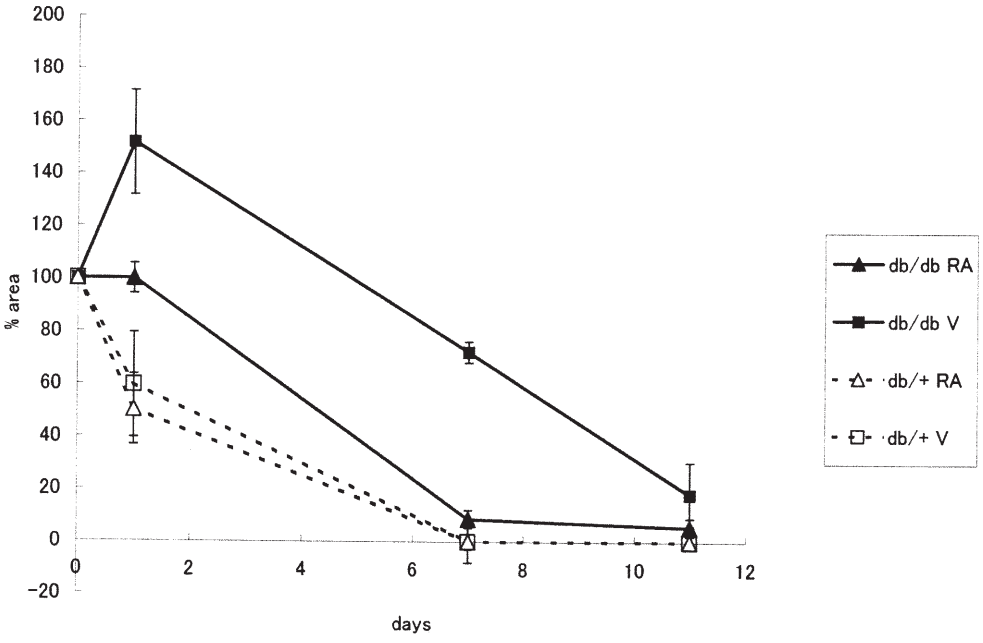

Fig. 4 Wound area of db/+ and db/db mice. Round wounds of diameter 6 mm were made on the back skin of mice pretreated with either 0.1% atRA or vehicle. Wounds of db/+ mice had almost closed by day 7 whether they had been treated with 0.1% atRA or vehicle. On the other hand, vehicle-treated db/db mice showed enlargement of the wound area on day 1, which was followed by delayed wound contraction. Pretreatment with atRA prevented the wounds of db/db mice from expanding, which may have contributed to the earlier wound closure. Data represent means ± SD of duplicate measurements from three independent experiments

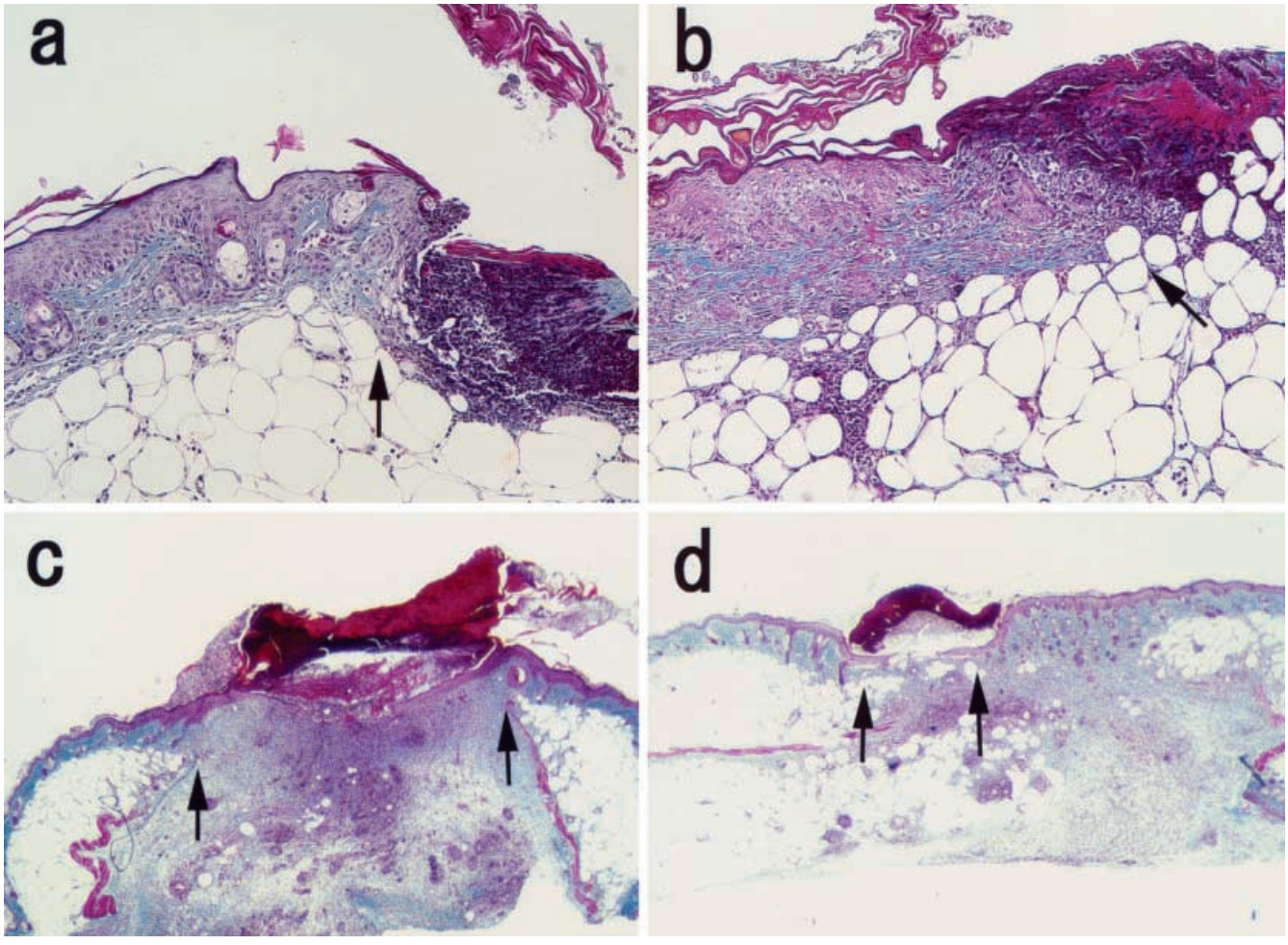

Fig. 5 a–c Wound sections of db/db mice pretreated with vehicle (**a**, **c**) or atRA (**b**, **d**) were stained with Gomori's trichrome. Arrows indicate the wound margin (**a**, **b** 1 day after wounding; **c**, **d** 14 days after wounding). **a**, **b** ×200; **c**, **d** ×50

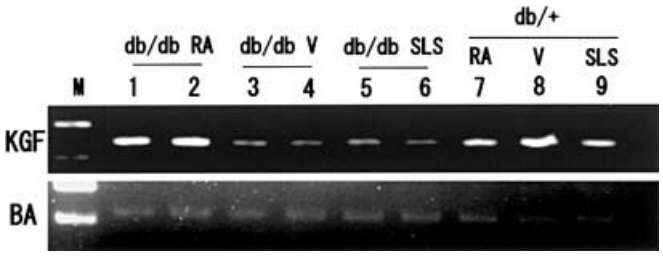

Fig. 6 Expression of KGF mRNA in day 1 wounds of db/db and db/+ mice treated with atRA (*RA*), sodium lauryl sulphate (*SLS*), or vehicle (*V*). A representative result of RT-PCR from three independent experiments is shown (*KGF* KGF mRNA, *BA* β-actin mRNA)

UVB-irradiated mice, but not in non-irradiated hairless mice [19, 20]. It has also been reported that atRA effectively reverses steroid-retarded repair, which may be a result of a mutual interaction between glucocorticoids and retinoids in the process of inflammation, immunity and connective tissue production [4–7]. It is interesting that the sebaceous glands of db/db mice showed extremely strong changes. Sebaceous glands almost completely disappeared in db/db mice after treatment with 0.1% atRA, but those in db/+ mice did not show significant changes after the same treatment. Even with 0.4% atRA, about 30% of sebaceous glands remained intact in db/+ mice, although significant dermal hypertrophy was observed with this concentration of atRA.

Pretreatment with 0.1% atRA had little effect on the wound healing in normal mice, but it significantly accelerated wound healing in db/db mice. This may reflect the biological effects of atRA pretreatment in these animals. Delayed wound healing in db/db mice seemed to be enhanced by treatment with atRA. First, full-thickness wounds in db/db mice had enlarged 20–40% just after wounding due to their extremely thin dermis with a small amount of extracellular matrix, which was the primary cause of delayed wound healing in db/db mice during the early stages of wound healing. Treatment with atRA made the dermis thick and stiff preventing enlargement of excisional wounds in db/db mice. Second, granulation tissue and extracellular matrix formation induced by atRA may also accelerate wound healing in db/db mice. This granulation tissue contains huge numbers of inflammatory cells that release cytokines and growth factors that enhance wound healing. In addition, smooth muscle actin fiber involved in wound granulation may exert a contractile force.

The mechanism of action of atRA in wound healing is complicated. A study using a dominant-negative retinoic acid receptor mutant strongly indicates that epidermal proliferation and regeneration is initiated by heparin-binding epidermal growth factor (HB-EGF) induced by atRA [21, 22]. Since atRA induction of HB-EGF occurs in suprabasal cells prior to the onset of basal cell hyperproliferation, this mechanism may well explain the effectiveness of atRA pretreatment. On the other hand, atRA may also enhance macrophage function, and thus finally may enhance macrophage-derived growth factors such as FGF, TGF and IGF-I [6]. This is also plausible since macrophage dysfunction is seen in db/db mice [23]. A more sensitive real-time RT-PCR could detect quantitative differences in these macrophage-derived growth factors.

Although suppression of KGF is characteristic of wound healing in db/db mice, KGF mRNA levels in unwounded skin are almost the same between db/db and db/+ mice [20], which was also confirmed by our RT-PCR (data not shown). In addition, atRA-induced epidermal proliferation in db/db mouse was not associated with KGF upregulation before wounding. Taken together, atRA does not directly induce KGF in unwounded skin but it causes the skin of db/db mice to induce KGF.

Previous studies of atRA application to already existing wounds have revealed conflicting results. Some investigators have shown enhanced healing [7, 14, 24], but others have shown retardation of healing [10, 25, 26]. On the other hand, topical pretreatment with atRA has consistently shown improvement in wound healing. In addition, our experimental and clinical experience has revealed that atRA worsens wound healing of full-thickness open wounds (unpublished data). For these reasons we do not recommend atRA application to diabetic ulcers. In practice, atRA may enhance wound healing when it is applied before surgery in diabetic patients. In the present study, we only evaluated the effects of atRA pretreatment in diabetic wounds, but it may be possible to apply this strategy to other healing-impaired wounds, such as those in the skin of the elderly or malnourished, or ischemic skin. Pretreatment with atRA may prevent delayed healing, infection and dehiscence in these potentially healing-impaired wounds.

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