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Effect of topical application of a stable prostacyclin analogue, SM-10902 on wound healing in diabetic mice

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Abstract

The mechanism of wound healing induced by topical application of an ointment containing a new stable prostacyclin analogue, SM-10902 ((+)-methyl[2-[(2R,3aS,4R,5R,6aS)-octahydro-5-hydroxy-4-[(E)-(3S,5S)-3-hydroxy-5-methyl-1-nonenyl]-2-pentalenyl] ethoxy] acetate), was investigated in the full-thickness wounds of genetically diabetic mice (*db/db* mice). The *db/db* mice treated with SM-10902 ointment (1, 10 and 100 μg/g) showed greater decrease in wound lesion area not covered with epidermis and fewer complete healing days than those treated with ointment base, and the effects of this prostacyclin analogue were greater than those of lysozyme chloride ointment (50 mg/g, Reflap ointment). SM-10902 ointment increased skin blood flow in the central site of the wound with development of wound healing. Histological evaluation of wounds revealed that SM-10902 ointment increased the capillary number during the early stage of the wound-healing process. These results suggest that SM-10902 ointment promotes wound healing through the stimulation of angiogenesis and the improvement of blood flow in neovascularization of repairing wound and may be useful in the treatment of skin ulcers caused by peripheral circulatory insufficiency.

Keywords: SM-10902 ointment; Wound healing; Prostacyclin analog; Blood flow, skin; Angiogenesis; Diabetic mouse (*db/db* mouse), genitically

1. Introduction

Prostacyclin and prostaglandin E_1 have been shown to be potent peripheral vasodilators (Moncada et al., 1976) and inhibitors of platelet aggregation (Bunting et al., 1976; Emmons et al., 1967), and have therapeutic potential in thrombotic diseases, such as peripheral vascular disease (Carlson and Erikson, 1973; Belch et al., 1983a, b). However, their clinical and experimental use have been limited by chemical and metabolical instability. In addition, when administered systemically, prostacyclin and prostaglandin E_1 are likely to cause side-effects, such as diarrhea, headache (Lewis et al., 1981), and 'steal' phenomenon (Koch-Weser and Coffman, 1979). To overcome these disadvantages, percutaneously active prostaglandin derivatives have been evaluated (Adachi et al., 1992; Uekama et al., 1992; Kecskes and Blitstein-Willinger, 1993).

SM-10902 ((+)-methyl[2-[(2R,3aS,4R,5R,6aS)-octahydro-5-hydroxy-4-[(E)-(3S,5S)-3-hydroxy-5-methyl-1-nonenyl]-2-pentalenyl] ethoxy] acetate) is a novel stable analogue of prostacyclin (Kawakami et al., 1993) and a prodrug with its vasodilation and antiplatelet activities expressed by its free acid metabolite, SM-10906 (Yamamoto et al., 1994), which have been shown to be an agonist for the prostacyclin receptor but not the prostaglandin E_2 receptor in mouse mastocytoma P-815 cells (Oka et al., 1994). Since SM-10902 penetrates into the skin much better than SM-10906 or prostacyclin (Yamamoto et al., 1994), it is expected to be useful as an external preparation for the treatment of skin ulcers caused by peripheral circulatory insufficiency.

Recently, topical application of recombinant human platelet-derived growth factor and of recombinant human basic fibroblast growth factor have been shown to be effective in accelerating impaired wound healing in an animal model with full-thickness skin wounds made on the backs of genetically diabetic mice (*db/db* mice) (Greenhalgh et al., 1990; Tsuboi and Rifkin, 1990; Matuszewska et al., 1994). The *db/db* mice are useful as an animal model for wound-healing studies since wound healing in these mice is markedly delayed when compared with

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nondiabetic littermates (Greenhalgh et al., 1990; Yamamoto et al., 1993; Matuszewska et al., 1994), and are reported to be relevant to the impaired healing observed in humans with diabetes mellitus, such as suppressed cell-mediated immunity and microvascular lesions (Mandel and Mahmoud, 1978; Coleman, 1982; Greenhalgh et al., 1990). Therefore, the effects of SM-10902 ointment on wound healing were investigated in *db / db* mice.

In a previous study, we found that topical application of the prostacyclin analogue SM-10902 dissolved in polyethylene glycol promoted wound healing in *db / db* mice (Yamamoto et al., 1993). However, the mechanism of this wound healing induced by topical application of SM-10902 is not well understood. In this study, it was suggested that topical application of SM-10902 ointment promoted wound healing through the stimulation of angiogenesis and the improvement of blood flow in neovascularization of the repairing wound.

2. Materials and methods

2.1. Materials

SM-10902 was synthesized in our laboratory (Sumitomo Pharmaceutical Co., Japan). SM-10902 ointment containing SM-10902 was adjusted to 1, 10 and 100 μg/g with ointment base containing an optimal dose of white petrolatum. Lysozyme chloride ointment (50 mg/g, Reflap ointment) was purchased from Hitachi Chemical Co. (Japan).

2.2. Animals

Genetically diabetic female C57BL/KsJ *db* + / *db* + mice (*db / db* mice) were obtained from Jackson Laboratory (Bar Harbor, USA). The *db / db* mice were housed twenty per cage with free access to water and food and maintained under controlled environmental conditions (12-h light/dark cycle, temperature 23 ± 2°C and humidity 55 ± 10%).

2.3. Wounding

After the hair on the back of *db / db* mice (about 10-11 weeks old) was clipped, the mice were housed in individual cages throughout the experimental period. After feeding for 1 week, the excised wound models were prepared by a modification of the method of Greenhalgh et al. (1990) using normal *db / db* mice weighing about 40–50 g. Briefly, the mice were anesthetized with ether, and the skin of the back of *db / db* mice was gently wiped and sterilized with ethanol. A full-thickness wound (1.5 cm square) was made by excising the skin. The transparent dressing Tegaderm (3M Canada, Canada), which shows good oxygen- and moisture vapor-permeability, was placed over the wound. Fifty mg of ointment was applied to each wound once a day through a cut on the Tegaderm until the wound lesion closed completely. The wound lesion area, which was not covered with epidermis, was measured from polyvinyl film tracings every 3 days with an image analyzer (System Supply Co., Japan). The wound lesion sizes were calculated against original area (on day 0) which was designated as 100%. The day when the full-thickness wound was closed completely by epidermis was regarded as the day of complete healing.

2.4. Measurement of skin blood flow

The *db / db* mice treated with SM-10902 (10 μg/g) ointment or its ointment base for 5, 10, 15 and 20 days were anesthetized with urethane (1.0 g/kg, s.c.). Stable basal levels of skin blood flow were measured in the central site of the wound with a laser-doppler flow meter (ALF 2100; ADVANCE Co., Japan). Skin blood flow was output in arbitrary units (A.U.) by an attached digital printer (ALF-P1; ADVANCE Co.). Then, 50 mg of SM-10902 ointment or its ointment base were applied to the wound, and changes in the skin blood flow were recorded at 5, 10, 20, 30, 40, 50 and 60 min after application.

2.5. Histological evaluation

After measurement of skin blood flow, the *db / db* mice were killed by bleeding from the common carotid arteries. The dorsal skin, including the wound site, was isolated and fixed with phosphate-buffered saline containing 10% formaldehyde. The tissue was embedded in paraffin, and a histological specimen penetrating the center of wound was made perpendicular to the anterior-posterior axis and the surface of the wound. Separate segments were stained with hematoxylin and eosin and processed further for light microscopic observation. The degree of granulation tissue thickness in the entire wound area, and the number of capillaries in the edge and central site of wounds were evaluated using masked slides by the method of Tsuboi and Rifkin (1990). Each of the parameters was graded numerically as described below.

Granulation tissue thickness. A value of 1 represents a thin granulation layer; 2, moderate granulation layer; 3, a thick granulation layer; 4, a very thick granulation layer.

Capillary number. The number of capillary lumens was counted on the edge and the central wound cross-section at ×25 magnification. A score of 0 represents 0–4 capillaries per site; 1, 5–9 capillaries per site; 2, 10–14 capillaries per site; etc.

The length of re-epithelialization and between the original outlines in a histological section was measured using the micrometer under the microscope at ×10 magnification.

2.6. Statistical analyses

Values were expressed as means ± S.E.M. Statistical analysis of wound lesion size and the complete healing

days was performed using Steel, Williams's or Shirley–Williams's multiple comparison after analysis of variance. The data of skin blood flow were compared between the groups using an unpaired Student's *t*-test or Welch's test. The data of the histological evaluations also were analyzed statistically using an unpaired Student's *t*-test or Mann–Whitney's *U*-test. Differences were considered to be significant if the *P* value was less than 0.05.

3. Results

3.1. Effect of SM-10902 ointment on wound lesion size

The initial wound area before application of nontreatment, SM-10902 ointment base, SM-10902, 1, 10, 100 μg/g ointment and lysozyme chloride ointment were 292.6 ± 9.2 ($n = 8$), 295.7 ± 9.1 ($n = 8$), 280.3 ± 8.6 ($n = 8$), 305.9 ± 9.8 ($n = 8$), 285.8 ± 11.3 ($n = 8$) and 279.4 ± 4.5 ($n = 8$) mm^2, respectively, there was not a significant difference among those groups. The wound lesion size of every group was closing with time. As shown in Fig. 1 and Table 1, there were significant reductions in wound lesion size and complete healing days in wounds treated with the ointment base of SM-10902 ointment when compared with those in nontreated wounds. The *db / db* mice treated with SM-10902 (10 or 100 μg/g) ointment had greater decrease in wound lesion size from 9 days after application than those treated with its ointment base (Fig. 1). Lysozyme chloride ointment also caused greater decrease in wound lesion size compared with the nontreatment group, but the effect of lysozyme chloride ointment was less than that of SM-10902 ointment. As shown in Table 1, SM-10902 ointment caused significant reduction in complete healing days in a concentration-dependent manner from 1 to 100 μg/g in comparison with the ointment base. Lysozyme chloride ointment also tended to reduce the complete healing days compared with the nontreatment group.

Table 1
Effect of SM-10902 ointment on the number of days required for complete healing in *db / db* mice

Treatment	Number of animals	Days to complete healing
Nontreatment	8	38.4 ± 1.8
Ointment base	8	25.3 ± 1.0 [c]
SM-10902 ointment		
1 μg/g	8	22.1 ± 0.9 [a,c]
10 μg/g	8	20.0 ± 0.8 [b,c]
100 μg/g	8	18.9 ± 0.8 [b,c]
Lysozyme chloride ointment	8	33.5 ± 2.0

Full-thickness skin wounds in *db / db* mice were treated with 50 mg/day of ointment base, SM-10902 ointment (1, 10 or 100 μg/g) or lysozyme chloride ointment. The number of days until complete closure of wounds was regarded as the complete healing day. Values are expressed as means ± S.E.M. Statistical significance: [a] $P < 0.05$ and [b] $P < 0.01$ vs. ointment base-treated *db / db* mice by Shirley–Williams's multiple comparison. [c] $P < 0.01$ vs. nontreated *db / db* mice by Steel's multiple comparison.

3.2. Effect of SM-10902 ointment on skin blood flow

Basal levels of blood flow at the central site of the skin wound in *db / db* mice treated with SM-10902 ointment or its ointment base were measured during the development of wound healing (Fig. 2). In *db / db* mice treated with 10 μg/g SM-10902 ointment, the basal blood flow tended to

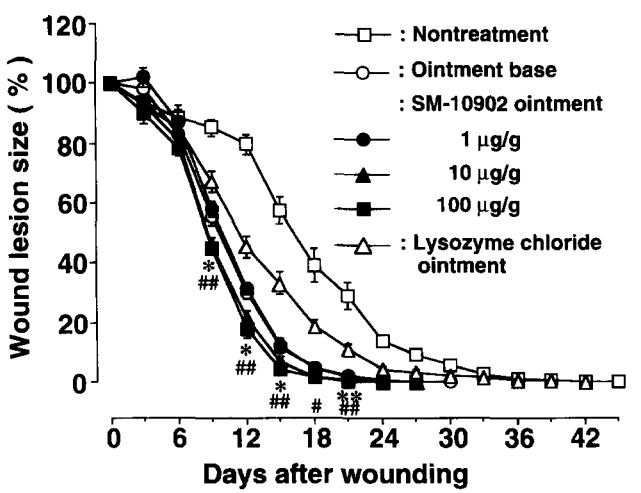

Fig. 1. Effect of SM-10902 ointment on excised wound healing in *db / db* mice. Full-thickness skin wounds in *db / db* mice were treated with 50 mg/day of ointment base, SM-10902 ointment (1, 10 or 100 μg/g) or lysozyme chloride ointment. Values are presented as percentages of the original wound area (mean ± S.E.M. of 8 animals). Statistical significance: * $P < 0.05$ and ** $P < 0.01$ (10 μg/g), # $P < 0.05$ and ## $P < 0.01$ (100 μg/g) vs. ointment base-treated *db / db* mice by Williams's or Shirley–Williams's multiple comparison.

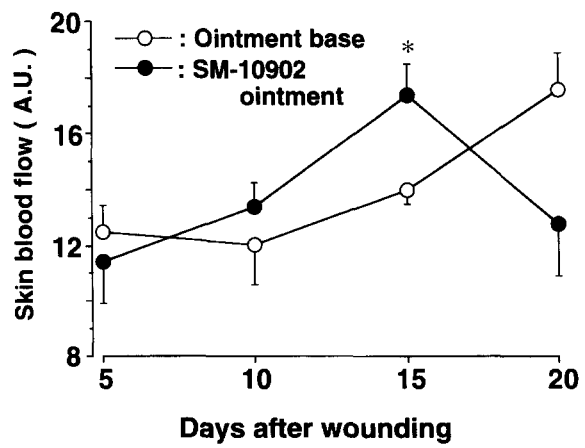

Fig. 2. Effect of SM-10902 ointment on skin blood flow before application of ointment to full-thickness wounds in *db / db* mice. Wounds in *db / db* mice were treated with 50 mg/day of SM-10902 ointment (10 μg/g) or ointment base for 5, 10, 15 and 20 days. Values are presented as volume of blood flow (mean ± S.E.M. of 5–6 animals). Statistical significance: * $P < 0.05$ vs. ointment base-treated *db / db* mice by Student's *t*-test for unpaired values.

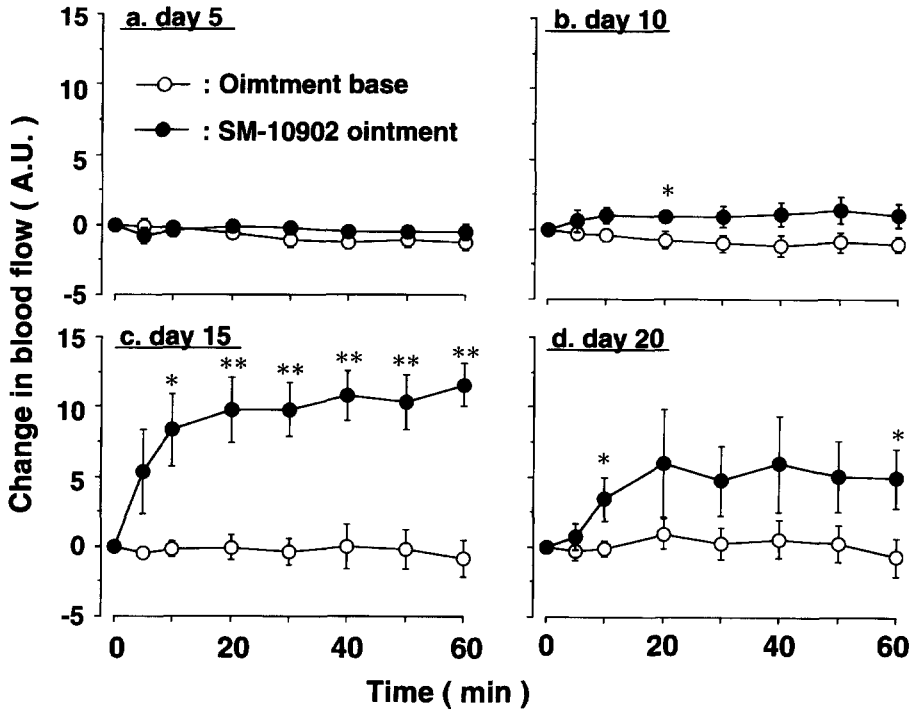

Fig. 3. Changes in the skin blood flow induced by SM-10902 ointment in full-thickness wounds of *db/db* mice. On days 5, 10, 15 and 20, the changes in skin blood flow at the center of wounds was measured following application of each ointment. Values are presented as changes in blood flow (mean ± S.E.M. of 5–6 animals). Statistical significance: * $P < 0.05$ and ** $P < 0.01$ vs. ointment base-treated *db/db* mice by Student's *t*-test for unpaired values.

increase on day 10, and was increased significantly compared with ointment base-treated *db/db* mice on day 15. On day 20, the basal blood flow tended to be reduced. In *db/db* mice treated with the ointment base, the basal blood flow tended to increase gradually from days 15 to 20. Thus, increased basal levels of skin blood flow were observed in SM-10902 ointment-treated *db/db* mice in the early stage of wound healing. In the edge site of the wound, we could not measure the blood flow because we had difficulty to steady the probe of a laser-doppler flow meter on there which had a differing and sloping surface.

Fig. 3 shows acute changes in the blood flow in the central site of the wound after the topical application of SM-10902 ointment at various time points. Ten μg/g of

Table 2
Time course of wound-healing in *db/db* mice treated with SM-10902 ointment or its ointment base

Treatment	Re-epithelialization (mm)	Original outline (mm)	Granulation tissue thickness (score)	Capillary number (score)	
				Central site	Edge site
Ointment base					
Days 5	0.4 ± 0.1	11.8 ± 0.3	1.0 ± 0.0	1.3 ± 0.3	4.0 ± 0.6
Days 10	0.9 ± 0.2	9.9 ± 0.8	1.7 ± 0.3	1.7 ± 0.3	4.0 ± 0.6
Days 15	1.9 ± 0.6	7.6 ± 1.0	2.7 ± 0.3	2.0 ± 0.0	3.0 ± 0.0
Days 20	3.8 ± 0.7	6.5 ± 0.3	3.7 ± 0.3	4.3 ± 1.2	3.0 ± 0.6
SM-10902 ointment					
Days 5	0.3 ± 0.1	11.7 ± 0.7	1.3 ± 0.3	1.8 ± 0.6	4.8 ± 1.3
Days 10	1.5 ± 0.1 [b]	8.7 ± 0.4	2.3 ± 0.3	2.8 ± 0.3	8.0 ± 0.4 [a]
Days 15	5.2 ± 0.5 [c]	6.0 ± 0.5	3.5 ± 0.3	3.8 ± 0.5 [a]	2.3 ± 0.5
Days 20	5.9 ± 0.3 [b]	5.9 ± 0.3	3.7 ± 0.3	3.3 ± 0.7	3.3 ± 0.3

Full-thickness skin wounds in *db/db* mice were treated with 50 mg/day of ointment base ($n = 3$) or SM-10902 ointment (10 μg/g; $n = 3$–4). Samples were taken at designated days. Scores of granulation tissue thickness and capillary number were graded as described in the text. The length of re-epithelialization and between the original outlines in a histological section was measured. Statistical evaluation was performed between SM-10902 ointment and ointment base treatments on the same days. Values are expressed as means ± S.E.M. Statistical significance: [a] $P < 0.05$ vs. ointment base-treated *db/db* mice by Mann–Whitney's *U*-test, [b] $P < 0.05$, [c] $P < 0.01$ vs. ointment base-treated *db/db* mice by Student's *t*-test for unpaired values.

SM-10902 ointments did not show any effect on day 5, but a tendency to increase was seen on day 10, and on day 15 a marked increase in the blood flow in the central site of wound was observed. On day 20, the increase in blood flow induced by topical application of SM-10902 ointment was slight. On the other hand, the topical application of the ointment base showed no effect on acute changes in the skin blood flow.

Fig.4

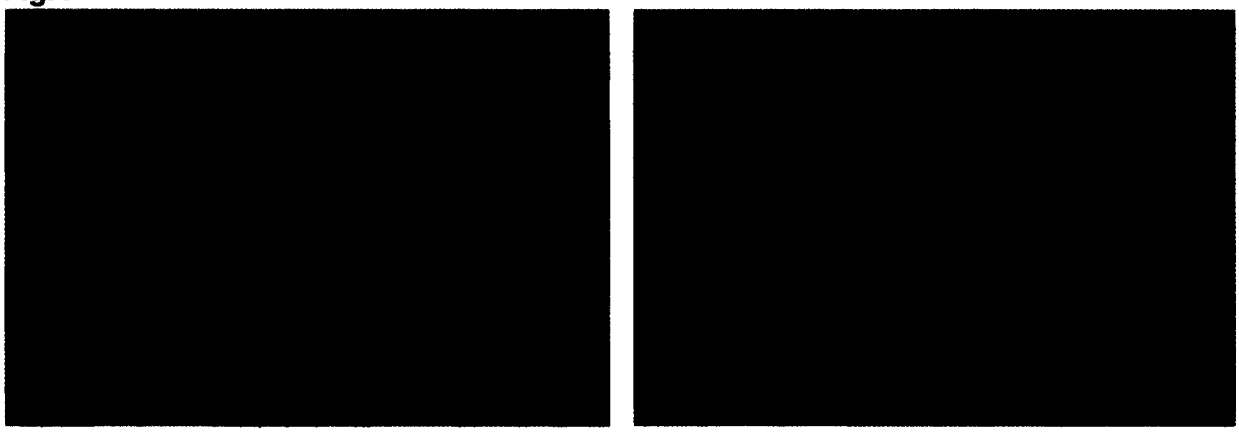

Fig.5

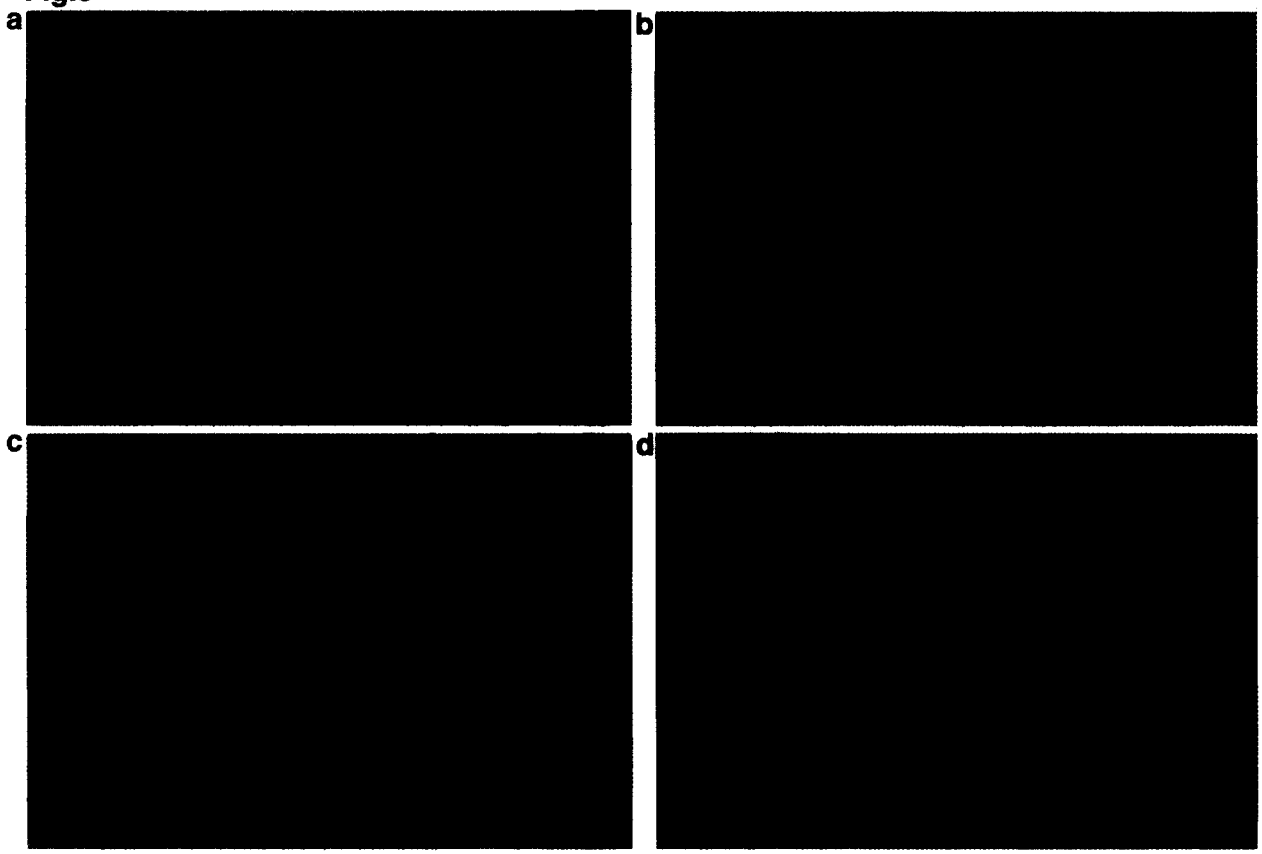

Fig. 4. Photomicrographs of specimens in the central wound sites of *db / db* mice treated with SM-10902 ointment or ointment base alone for 15 days. a: Full-thickness wounds were treated with 50 mg/day of ointment base for 15 days. b: Full-thickness wounds were treated with 50 mg/day of SM-10902 ointment (10 μg/g) for 15 days. Histological specimens were stained with hematoxylin and eosin. ×25.

Fig. 5. Photomicrographs of specimens at the edge of wounds in *db / db* mice treated with SM-10902 ointment or ointment base alone on day 5 or 15. a,b: Full-thickness wounds were treated with 50 mg/day of ointment base alone (a) or SM-10902 ointment (10 μg/g; b) for 5 days. c,d: Full-thickness wounds were treated with 50 mg/day of ointment base (c) or SM-10902 ointment (d; 10 μg/g) for 15 days. Histological specimens were stained with hematoxylin and eosin. ×25.

3.3. Histological evaluation

On day 5, the dermal layers in the wounds treated with SM-10902 ointment or its ointment base were occupied with adipose tissue. On day 15, the formation of re-epithelialization and granulation tissue were promoted by the topical application of SM-10902 ointment (Fig. 4). Namely, the degree of re-epithelialization and granulation tissue thickness in wounds treated with SM-10902 ointment exceeded those in wounds treated with ointment base alone (Table 2).

The effect of SM-10902 ointment on the capillary numbers in the wounds was studied. On day 5, there were a few thrombi at the edge sites of the wounds on histological specimens of tissues treated with SM-10902 ointment or its ointment base (Fig. 5a,b). Numerous capillaries, endothelial cells, macrophages and fibroblasts around thrombi at the edge sites of the wounds were observed in those treated with SM-10902 ointment (Fig. 5b), but these findings were much less marked in wounds treated with ointment base alone (Fig. 5a). On day 15, these histological phenomena observed in the wounds treated with SM-10902 ointment seemed to shift from the edge to the central site of the wound (Fig. 5). Namely, the increase in capillary number in the central site of the wounds treated with SM-10902 ointment occurred later than those at the edge site (Table 2).

On the other hand, there were no obvious differences between the contractions of wounds treated with SM-10902 ointment and its ointment base at every point (Table 2).

4. Discussion

Homozygous *db/db* mice are useful as an animal model of wound healing which is markedly delayed when compared with their heterozygous, nondiabetic littermates (Greenhalgh et al., 1990; Yamamoto et al., 1993; Matuszewska et al., 1994). Using the model of wound healing in *db/db* mice, we reported previously that topical application of SM-10902 dissolved in polyethylene glycol promoted wound healing (Yamamoto et al., 1993).

In this study, the effect of SM-10902 ointment on excised wound healing was investigated in *db/db* mice. SM-10902 ointment reduced complete healing days and wound lesion size significantly, and its action was available at concentrations up to 1 μg/g compared with the ointment base alone. The effects of ointment containing 1 μg/g SM-10902 on decrease in wound lesion size and complete healing days were more potent than those of lysozyme chloride ointment (Fig. 1, Table 1). Topical application of the ointment base or lysozyme chloride ointment also showed greater decrease in the wound lesion size and fewer complete healing days than the nontreatment group. The main ingredient of the SM-10902 ointment base is white petrolatum which is used clinically as a wound dressing and packing material. Ointment gauzes containing white petrolatum have been used as wound dressings with promotion of wound healing in Japan. The proliferative responses of fibroblasts and keratinocytes to lysozyme chloride in culture were reported by Takaoka et al. (1972) and Ishida et al. (1989), respectively. The usefulness of lysozyme also has been demonstrated in experimental models of incised wounds, open wounds and scalding (Kitoh et al., 1994). Therefore, our results obtained in this study were supported by the above effects of white petroleum and lysozyme chloride. From these results, we demonstrated that SM-10902 ointment as well as SM-10902 solution promoted wound healing macroscopically.

While the relationship with wound contractions in wound closure in the *db/db* mice treated with each ointment was difficult to assess on account of an unclear original outline of the wounds in every group during experimental period macroscopically, but no obvious differences between the contraction of wounds treated with SM-10902 ointment and its ointment base were observed from the histological evaluation (Table 2). SM-10902 ointment also stimulated the formation of re-epithelialization and granulation tissue greater than its ointment base (Table 2). Therefore, it is suggested that the response of wound closures by SM-10902 ointment in the *db/db* mice occurred as a result of the formation of re-epithelialization and granulation tissue.

On the other hand, there were no differences among the body weight of every group during the experimental period (data not shown), and no significant changes were observed on the systemic arterial pressure after topical application of SM-10902 ointment to the full-thickness skin wound or stripping skin in the rabbit ears (Hara et al., 1995; Yamamoto et al., 1996) or to the femoral skin in rats (Komuro et al., 1995). Our results suggest that topical application of SM-10902 ointment to the wound exerts no systemic action.

SM-10906 was shown to cause vasodilation and to have antiplatelet activity (Yamamoto et al., 1994). Moreover, SM-10902 was shown to penetrate well into the skin (Yamamoto et al., 1994). In this study, SM-10902 ointment caused a marked acute increase in skin blood flow in the central site of wounds with the development of wound healing (Fig. 3). The basal levels of skin blood flow in SM-10902 ointment-treated *db/db* mice also increased gradually on days 10 and 15. Thus, increases in basal blood flow in SM-10902 ointment-treated *db/db* mice were observed at an early stage of wound healing (Fig. 2). These results suggest that SM-10902 ointment may stimulate improvement of the blood flow in wounds due to vasodilation and antiplatelet activity induced by SM-10906.

On day 20, however, the basal blood flow and the acute increase in blood flow in wounds treated with SM-10902 ointment were slight (Fig. 2, Fig. 3). Tissue responses to injury have generally been divided into three main overlap-

ping phases: inflammation, granulation tissue formation, and matrix formation and remodeling (Clark, 1985). One of the most important stages in the process of wound healing is angiogenesis, which is induced with development of granulation tissue formation, and neovasculature induced by angiogenesis is reduced by matrix formation and remodeling (Clark, 1985). Tsuboi and Rifkin (1990) also reported an increase in capillary number were induced by recombinant basic fibroblast growth factor with development of wound healing, but these were reduced by matrix formation and remodeling in the final phase of the wound-healing process. In this study, since the period required for complete wound healing in *db / db* mice treated with SM-10902 ointment was about 20 days (Table 1), the slight increase in blood flow in *db / db* mice treated with SM-10902 ointment on day 20 may result from a decrease in capillary number by matrix formation and remodeling of wound-healing process.

In normal back skin of the *db / db* mice, topical application of SM-10902 ointment did not induce increase in the blood flow, and there were the thin epidermis layer and not many capillaries in the dermis layer filled by adipose tissue histologically (data not shown). Bohlen and Niggl (1979) also reported that the arterioles in *db / db* mice are characterized by a decreased number of arterioles, loss of vascular tone and a reduced cross-sectional area in the vessel walls. While the normal rat femur skin and rabbit ear skin treated with SM-10902 ointment were observed with continuous increase in the skin blood flow (Komuro et al., 1995; Hara et al., 1995; Yamamoto et al., 1996). Therefore, in the normal back skin of *db / db* mice, not the normal rats or rabbits, it may be difficult for SM-10902 ointment to increase the blood flow due to microvascular lesions. As our results obtained in blood flow studies must be assessed carefully due to alterations in the structure/density of the wound with time, we made an attempt at the approach of the histological evaluation.

In the histological specimens of the edges of wounds, numerous capillaries, endothelial cells, macrophages and fibroblasts around thrombi were observed in *db / db* mice treated with SM-10902 ointment on days 5 and 10 (Fig. 5b). Namely, increases in capillary number induced by SM-10902 ointment were significant compared with those induced by its ointment base at the edges of wounds on day 10 (Table 2). Prostaglandin E series have been shown to have angiogenic activity in the rabbit corneal test or the chick embryo chorioallantoic membrane assay (Form and Auerbach, 1983; Ziche et al., 1982; Benezra, 1978). However, prostacyclin showed no angiogenic activity (Ziche et al., 1982; Ohtsu et al., 1988). Recently, the stable prostacyclin analogues, isocarbacyclin and 7-fluoro prostacyclin, which have similar pharmacological properties to naturally occurring prostacyclin except in terms of chemical stability, exhibited angiogenic activity in the chick embryo chorioallantoic membrane assay (Ohtsu et al., 1988). As SM-10906, which is converted from SM-10902 by enzymic hydrolysis by esterase, is known to be an agonist of the prostacyclin receptor in mouse mastocytoma P-815 cells (Oka et al., 1994), topical application of SM-10902 ointment to wounds might stimulate angiogenesis similarly to prostaglandin E series and prostacyclin analogues. Furthermore, the numerous capillaries observed in the wounds treated with SM-10902 ointment seemed to shift from the edge to the central site of the wound, the increase in a number of capillaries obtained from histological evaluation gave agreement with those in the basal level of blood flow in the central site of wound during the development of wound healing (Fig. 4b, Table 2). These data indicated that an increase in the basal level of the blood flow resulted from an increase in the number of capillaries in wound. Therefore, it was suggested that topical application of SM-10902 ointment to wounds in presence of numerous capillaries might induce an increase in acute blood flow. Hereafter, it is necessary to study angiogenesis induced by SM-10902 in vitro.

On the other hand, the formation of granulation tissue and re-epithelialization were observed in the wound treated with SM-10902 ointment (Fig. 4b, Table 2). Zhang et al. (1988) reported that several activators of adenylate cyclase, such as prostaglandin E_1, forskolin and the phosphodiesterase inhibitor isobutylmethylxanthine, evoked increases in interleukin-6 mRNA levels in human fibroblasts. Grossman et al. (1989) also reported that interleukin-6 stimulates proliferation of cultured human keratinocytes. Furthermore, it was shown that interleukin-6 induced by SM-10902 and SM-10906-stimulated cyclic adenosine 3′,5′-monophosphate formation in human dermal fibroblasts stimulated proliferation of fibroblasts and keratinocytes (Kaneko et al., 1995). Therefore, our results obtained by histological evaluation suggested that topical application of SM-10902 ointment in vivo might promote a formation of granulation tissue and re-epithelialization by the proliferation of fibroblasts and keratinocytes directly. Interestingly, many endothelial cells and fibroblasts were observed around the numerous neovasculature (Fig. 5b). Blood containing high levels of oxygen and nutrients flow into the neovasculature in the wound, and these have been shown to promote wound healing (Barbara and Clark, 1985; Heng, 1983; Fischer, 1969). Therefore, as SM-10902 ointment caused an increase in blood flow in the wound, we speculated that this prostacyclin analogue might also stimulate the proliferation of fibroblasts and endothelial cells through improvement of the blood flow.

In summary, these results suggest that ointment containing the prostacyclin analogue SM-10902 may promote wound healing through the stimulation of angiogenesis and an improvement of skin blood flow in neovasculature in wounds of *db / db* mice. Therefore, SM-10902 ointment may be useful as an external preparation to improve skin ulcers caused by peripheral circulatory insufficiency.

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