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Wound healing acceleration of a novel transforming growth factor-β inducer, SEK-1005

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Abstract

The studies were carried out to elucidate the effect of a novel cyclic peptide, SEK-1005 ($C_{45}H_{70}N_8O_{13}$), on wound healing. SEK-1005 (4–10 μg/wound) applied topically significantly accelerated the healing of a full-thickness wound on the dorsal skin of a rat. In a healing-impaired mouse, the peptide (2–10 μg/wound) had more potent activity, exerting an effect comparable to that of basic fibroblast growth factor (FGF). However, SEK-1005 (0.1–100 ng/ml) scarcely promoted the proliferation of cultured fibroblasts (NIH3T3 cells) while basic FGF (0.2–5 ng/ml) showed marked mitogenic activity. SEK-1005 (2–10 μg/wound) significantly increased the topical production of transforming growth factor (TGF)-β1, a cytokine that is known to accelerate wound healing. This activity was closely correlated with the wound-repairing effect. From the above, SEK-1005 can be considered as a new type of wound healing agent with potent TGF-β1-inducing activity.

Keywords: Wound healing accelerator; Healing-impaired, mouse; Cyclic peptide; SEK-1005; TGF-β1 (transforming growth factor-β1) inducer

1. Introduction

Acute skin wounds heal by means of a complex sequential process involving various cell types (Roberts, 1995). The failure of wounds to heal is a critical problem, especially in patients with an impaired healing response, such as patients with chronic diseases or in elderly individuals. There is a clinical need for an agent that will promote the healing of chronic wounds including diabetic ulcers or decubitus, and also for the prophylactic treatment of surgical patients with a predictably impaired healing response. Indeed, a few new types of preparations, such as a basic fibroblast growth factor (FGF) (Okumura et al., 1996a,b,c; Tanaka et al., 1996) and a prostacyclin analogue (Yamamoto et al., 1996), have been developed.

Based on the pathogenesis of wound healing, it has been suggested that chronic wounds may be deficient in an orderly production of cytokines (Falanga et al., 1994). Of the cytokines shown to affect wound healing, transforming growth factor (TGF)-βs with their broad activity are the principal cytokines involved in the repair of injured tissue, in addition to basic FGF. Indeed, some reports have demonstrated a therapeutic effect of TGF-βs on poorly healing wounds (Pierce et al., 1989; Quaglino et al., 1991; Chen et al., 1992; Roberts, 1995). However, it is not excluded that exogenous TGF-βs may disturb the orderly sequence of repair, compared with endogenous cytokines. SEK-1005 ($C_{45}H_{70}N_8O_{13}$, Fig. 1) is a novel cyclic peptide that was isolated from Streptmyces nobilis during a search for a new type of anti-inflammatory agent (Kuriyama et al., 1994, 2000; Abe et al., 1996). We now demonstrate that SEK-1005 is a potent inducer of TGF-β1 and promotes wound healing.

2. Materials and methods

2.1. Animals

Male Wistar rats or genetically diabetic female mice (C57BL/KsJ db + /db +)(db/db mice) were purchased from Nihon SLC (Hamamatsu, Japan) or Clea Japan (Tokyo, Japan), respectively. The animals were housed for

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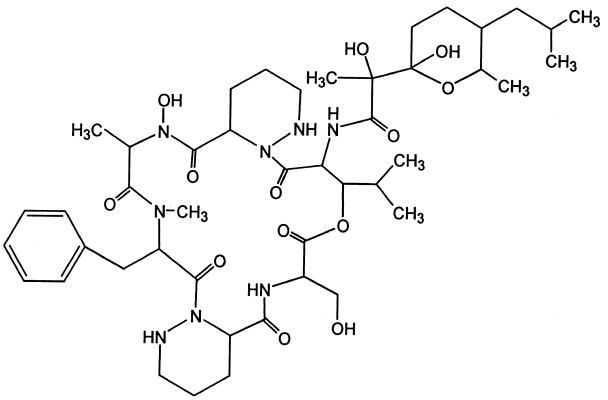

Fig. 1. Chemical structure of SEK-1005: Ser, 3-hydroxy-*N*-[2-hydroxy-1-oxo-2-tetrahydro-2-hydroxy-6-methyl-5-(2-methylpropyl)-2*H*-pyran-2-yl-propyl]-Leu-Pip(hexahydro-3-pyridazinecarbonyl)-*N*-hydroxy-Ala-*N*-methyl-Phe-Pip-,.rho.-lactone.

at least 7 days in this laboratory after their arrival. Constant temperature and humidity (22 ± 1°C, 55 ± 10%) were maintained with a fixed 12-h light–dark cycle and free access to food and water. Guiding principles for the care and use of laboratory animals approved by The Japanese Pharmacological Society were followed in this animal study.

2.2. Compounds

SEK-1005 [purity: more than 96% by UV detection at 210 nm after reverse-phase high-performance liquid chromatography; content of endotoxins: less than 1 EU/g by a commercial limulus assay (Endospecy) (Seikagaku, Tokyo, Japan)] was prepared from cultured Streptomyces nobilis in our laboratory as described previously (Fujiwara et al., 1999). The compound was suspended in 0.5% carboxymethyl-cellulose solution for the wound healing experiments and was dissolved in methanol for the cell culture experiments. Recombinant human basic FGF (Progen, Heidelberg, Germany) was dissolved in saline.

2.3. Wounding

A round full-thickness wound having a diameter of about 1.6 cm was made with scissors on the clipped dorsal skin of an animal under diethylether anesthesia. The wound was covered with a transparent occlusive dressing, Bioclusive (Johnson and Johnson, Arlington, TX, USA). The dressing was replaced every 2–3 days to collect the exudate and to measure the wound diameter after rinsing with saline. The curative effect is expressed relative to the wound area on day 0 (100%). It was calculated from the product of cross diameters of the wound site. The compounds to be tested were topically applied once in a volume of 20 μl/site and were present for about 48 h, until the dressing was changed. A corresponding amount of vehicle was given to control animals.

2.4. Cell culture

Mouse fibroblasts, NIH3T3 cells, were purchased from Dainippon Pharmaceutical (Osaka, Japan). The cells were plated at a density of 10^4 cells/well on 96-well microtiter plates (Corning Costar Japan, Tokyo, Japan) and incubated at 37°C in 100 μl of Dulbecco's minimum essential medium containing 0.1% fetal calf serum and the compound to be tested. After 16 h, 10 μl of [^{3}H]thymidine in saline (400 kBq/ml)(Amersham Pharmacia Biotech, Tokyo, Japan) was added to each of the wells and further incubated. Eight hours later, the intensity of radioactivity in the cells was measured to evaluate the effect on cell proliferation.

2.5. Measurement of TGF-β1 in wound exudate

Wound exudate was taken from female db/db mice during wound healing and centrifuged at $1000 \times g$ to separate the supernatant. The content of TGF-β1 in the supernatant was measured with an enzyme immuno-assay (TGFβ1 E_{max} Immuno Assay System, Promega, Madison, USA).

2.6. Statistical analysis

The data are presented as the means ± S.E.M. Statistical analysis was performed with Dunnett's multiple comparison test and P values < 0.05 were considered to be significant.

3. Results

3.1. Effect on full-thickness skin wound in rats

A full-thickness wound was made on the dorsal skin of male Wistar rats weighing 250–280 g. The wound area

Table 1
Effects of SEK-1005 and basic FGF on the healing of a full-thickness wound in rats

Compound	Dose (μg/wound)	Relative wound area (%) on day			
		2	4	5	7
Control	–	83.5 ± 2.8	63.1 ± 2.7	49.0 ± 3.0	16.5 ± 6.5
SEK-1005	4	75.6 ± 2.4	57.5 ± 1.8	45.7 ± 0.8	15.3 ± 4.8
	10	75.4 ± 2.5[a]	56.1 ± 2.8	41.0 ± 1.6[a]	14.9 ± 4.8
Control	–	79.2 ± 2.3	56.7 ± 2.0	43.0 ± 1.7	14.5 ± 4.5
Basic FGF	2	82.8 ± 3.8	59.7 ± 2.8	51.3 ± 3.2[a]	18.6 ± 2.8

Each figure indicates the mean ± S.E.M. for six animals. The mean wound area was about 2.1 cm^2 on day 0.

[a] $P < 0.05$: significant relative to the control.

Table 2
Effects of SEK-1005 and basic FGF on the healing of a full-thickness wound in db/db mice

Compound	Dose (μg/wound)	Relative wound area (%) on day					
		2	5	7	9	12	14
Control	–	101.1 ± 2.9	95.6 ± 3.2	93.1 ± 2.4	87.4 ± 2.7	79.1 ± 3.1	75.1 ± 3.9
SEK-1005	2	94.7 ± 2.7	87.6 ± 3.4	80.5 ± 6.2[a]	71.6 ± 8.2[a]	58.6 ± 16.4	46.2 ± 20.2[a]
	4	93.1 ± 1.4[a]	81.1 ± 1.3[b]	71.5 ± 0.8[a]	57.3 ± 2.2[b]	35.3 ± 2.1[b]	18.0 ± 4.9[b]
	10	94.8 ± 0.9	81.2 ± 1.8[b]	71.8 ± 1.1[b]	60.1 ± 1.6[b]	44.7 ± 3.9[b]	33.0 ± 4.2[b]
Control	–	101.7 ± 2.6	99.0 ± 2.1	91.0 ± 2.1	82.3 ± 2.1	80.2 ± 4.2	76.8 ± 2.9
Basic FGF	2	92.9 ± 1.7[a]	85.4 ± 2.9[b]	77.3 ± 3.9[b]	69.8 ± 5.6[a]	60.9 ± 8.9	57.9 ± 9.1

Each figure indicates the mean ± S.E.M. for five animals. The mean wound area was about 2.6 cm^2 on day 0.
[a] $P < 0.05$: significant relative to the control.
[b] $P < 0.01$: significant relative to the control.

was measured on days 0, 2, 4, 5 and 7. SEK-1005 and basic FGF were applied just after the wound was made on day 0. Table 1 shows the time course of changes in the relative wound area. The wound rapidly healed with time in the control, as the relative wound area was 79.2–83.5% on day 2 and 14.5–16.5% on day 7. In this model, although SEK-1005 in a dose of 10 μg/wound caused a significant acceleration of healing on days 2 and 5, it was a weak accelerator. A reference agent, basic FGF (2 μg/wound), which is reported to stimulate wound healing in healing-impaired mice (Greenhalgh et al., 1990; Tsuboi and Rifkin, 1990; Okumura et al., 1996a), had no accelerative activity in this normal rat model.

3.2. Effect on full-thickness skin wound in healing-impaired mice

A full-thickness wound was made on the dorsal skin of female db/db mice weighing 35–55 g. The wound area was measured on days 0, 2, 5, 7, 9, 12 and 14 or 16. Table 2 shows the time course of changes in the relative wound area. The wound healed very slowly in db/db mice, compared with the result in rats (Table 1). The relative wound area was still 75.1–76.8% even on day 14, consistent with the report of Okumura et al. (1996a,c).

SEK-1005 and basic FGF were topically applied just after the wound was made to reveal their activities in this healing-impaired animal model. SEK-1005 produced a significant acceleration of wound healing in doses of 2–10 μg/wound, showing an effect nearly equal to that of basic FGF in a dose of 2 μg/wound, a dose which is reported to produce a maximum effect (Okumura et al., 1996a,c). The time to 50% wound closure was 14 days for 2 μg/wound and 12 days for 4 and 10 μg/wound while it was more than 14 days in the control. Moreover, from the microscopic findings in a preliminary study, the acceleration of wound closure induced by SEK-1005, seems to be mainly due to an increase in the rate of re-epithelialization following the formation of granulation tissue.

The experiment shown in Table 3 was carried out to study the relationship between the wound healing activity and the dosing time. SEK-1005 (4 μg/wound) was applied just after or 7 days after the wound was made. SEK-1005 was active at both dosing times. Thus, SEK-1005 applied on day 7 caused a significant acceleration of wound healing on days 9–16, having a similar time course of healing to that of when it was applied on day 0. These findings suggest that SEK-1005 might be active over a broad time zone in the wound-closing process.

3.3. Effect on the proliferation of cultured fibroblasts

The mitogenic activity of SEK-1005 was studied using cultured mouse fibroblasts, NIH3T3 cells (Table 4). The

Table 3
Relationship between the SEK-1005 effect and the dosing time in db/db mice

Compound	Dose timing (on day)	Relative wound area (%) on day					
		2	5	7	9	12	16
Control	0	97.6 ± 2.9	93.2 ± 3.2	90.1 ± 1.9	83.8 ± 2.2	75.8 ± 2.3	64.0 ± 1.6
SEK-1005	0	89.5 ± 2.0	75.9 ± 4.2[a]	70.1 ± 2.0[b]	58.8 ± 2.1[b]	37.3 ± 3.3[b]	22.3 ± 8.4[a]
Control	7	106.8 ± 4.0	100.9 ± 4.5	93.9 ± 1.7	85.7 ± 1.7	75.1 ± 2.5	66.9 ± 1.8
SEK-1005	7	102.5 ± 2.8	98.4 ± 1.4	95.1 ± 1.4	75.0 ± 3.1[a]	53.8 ± 7.1[a]	35.0 ± 10.5[a]

Each figure indicates the mean ± S.E.M. for four to five animals. The mean wound area was about 2.4 cm^2 on day 0.
[a] $P < 0.05$: significant relative to the control.
[b] $P < 0.001$: significant relative to the control.

Table 4
Effects of SEK-1005 and basic FGF on the proliferation of cultured fibroblasts, NIH3T3 cells

Compound	Concentration (ng/ml)	Incorporation of [^{3}H] thymidine (cpm)
Control	–	457 ± 73
SEK-1005	0.1	663 ± 56
	1	769 ± 168
	10	512 ± 152
	100	601 ± 65
Control	–	413 ± 68
Basic FGF	0.2	761 ± 74[a]
	1	1008 ± 169[b]
	5	1472 ± 73[b]

Each figure indicates the mean ± S.E.M. for six wells.
[a] $P < 0.05$: significant relative to the control.
[b] $P < 0.01$: significant relative to the control.

cultured cells slightly proliferated under control conditions. A reference compound, basic FGF, significantly promoted cell proliferation and produced dose-dependent mitogenic activity in concentrations of 0.2–5 ng/ml. However, SEK-1005 scarcely showed mitogenic activity in concentrations of 0.1–100 ng/ml, and morphological cell damage was observed with the highest concentration (100 ng/ml). These results indicate that SEK-1005, unlike basic FGF, does not accelerate directly the proliferation of cultured fibroblasts.

3.4. Effect on the production of TGF-β1 in the wound exudate

The effect of SEK-1005 on TGF-β1 production in wound exudate was studied in female db/db mice weighing 45–55 g during wound healing. SEK-1005 was topically applied just after the wound was made on day 0. The amount of TGF-β1 in wound exudate was measured on days 2, 7 and 14. Table 5 shows the time course of changes in SEK-1005 activity. SEK-1005 significantly increased the amount of TGF-β1 in wound exudate in doses of 2–10 μg/wound, whereas TGF-β1 was undetected in exudate of the control. This activity was potentiated with time after wounding: there was a successive increase in cytokine production on days 2–14.

Table 5
Effect of SEK-1005 on TGF-β1 in wound exudate in db/db mice

Compound	Dose (μg/wound)	Amount of TGF-β1 (ng/wound) on day		
		2	7	14
Control	–	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
SEK-1005	2	0.34 ± 0.10[a]	0.56 ± 0.21[a]	0.64 ± 0.41[b]
	4	0.34 ± 0.07[a]	0.81 ± 0.12[a]	1.43 ± 0.24[a]
	10	0.16 ± 0.06[a]	0.94 ± 0.23[a]	1.46 ± 0.18[a]

Each figure shown as the accumulative sum from day 0 indicates the mean ± S.E.M. for three to five animals.
[a] $P < 0.01$: significant relative to the control.
[b] $P < 0.05$: significant relative to the control.

In this experiment, the wound area was also measured to define the contribution of TGF-β1 to wound healing. The value of each animal is plotted in Fig. 2. The relative wound area was demonstrated to be inversely correlated with the amount of TGF-β1 in wound exudate on days 7 and 14. These results suggest that the increased production of TGF-β1 induced by SEK-1005 might play an important role in its acceleration of wound healing.

4. Discussion

The experiments were carried out to elucidate the effect of SEK-1005 on wound healing, and the compound was

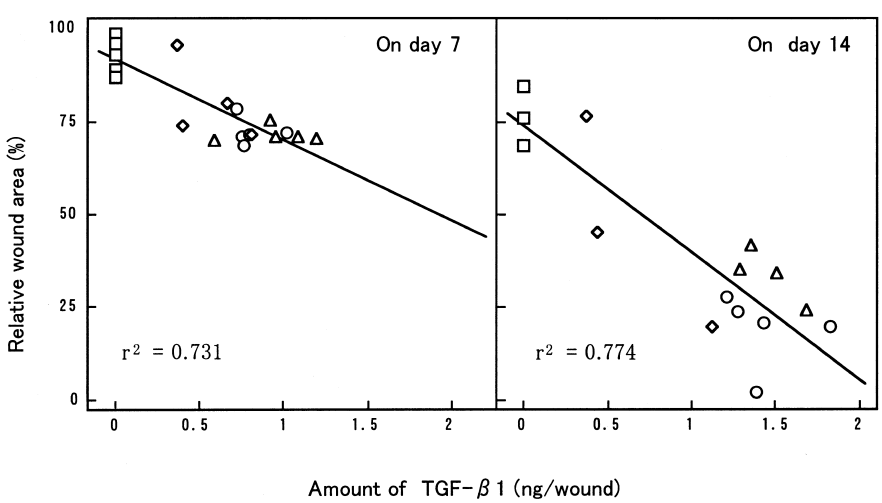

Fig. 2. A correlation between the relative wound area and the amount of TGF-β1 in wound exudate. The amount of TGF-β1 is presented as the accumulative sum from day 0. The mean wound area was about 2.6 cm^2 on day 0. □: Control, ◇: SEK-1005 2 μg/wound, ○: SEK-1005 4 μg/wound, △: SEK-1005 10 μg/wound.

demonstrated to be an excellent accelerator of wound healing.

A wound healing process can be categorized as follows: inflammation, granulation tissue formation, re-epithelialization and tissue remodeling (Martin, 1997). Especially, the inflammation response is considered to be a key process during which growth factors including TGF-β1 and platelet-derived growth factor (PDGF) are released from macrophages and platelets to initiate granulation tissue formation (Roberts, 1995; O'Kane and Ferguson, 1997). The immunological depression due to disorders and aging reduces the release of growth factors (O'Kane and Ferguson, 1997) and causes a delay of wound healing (Falanga, 1993). Therefore, the pharmacological effects of wound healing accelerators have been often evaluated in immuno-deficient models (Okumura, 1996b) induced by overdosing of steroids, experimental diabetes or nutrient limitation. Additionally, recent reports have demonstrated that genetically diabetic db/db mice with suppressed cell-mediated immunity (Mandel and Mahmound, 1978) are useful to test wound healing accelerators such as basic FGF (Tsuboi and Rifkin, 1990; Okumura et al., 1996a,b,c; Tanaka et al., 1996) and a prostacyclin analogue (Yamamoto et al., 1996). The inhibition of macrophage infiltration of inflammatory lesions in db/db mice is considered to induce the impairment of granulation tissue formation (Tsuboi and Rifkin, 1990).

In this paper, the effect on full-thickness wound was evaluated in two animal models. Although SEK-1005 showed only weak accelerating activity for wound healing in the normal rat model, it was more active and produced a marked acceleration of healing in the healing-impaired db/db mouse model mentioned above. These results suggest that SEK-1005 could restore the suppressive production of growth factors to initiate granulation tissue formation. In addition, according to the microscopic characterization in the db/db mouse model reported by Tsuboi and Rifkin (1990), granulation tissue is formed in two phases, namely, days 0–5 post wounding and the later period. SEK-1005 was effective with a single dose on either day 0 or 7, indicating that the efficacy of SEK-1005 is unaffected by the phase of granulation tissue formation.

Further studies were carried out to elucidate the possible role of SEK-1005 in the acceleration of wound healing. Cell proliferation was studied using cultured fibroblasts, NIH3T3 cells. SEK-1005 had little effect on cell proliferation, whereas basic FGF apparently promoted the proliferation of fibroblasts, as reported by Muller et al. (1984). SEK-1005 was found to be a potent inducer of TGF-β1, a cytokine competent for wound healing. The cytokine has been reported to play a therapeutic role involving the migration of inflammatory cells, the proliferation synthesis of collagen by fibroblasts and remodeling. It also interacts with other growth factors such as basic FGF, epidermal growth factor, keratinocyte growth factor and PDGF (Roberts et al., 1985; Ignotz and Massague, 1986; Roberts et al., 1986; Martin, 1997). Topical SEK-1005 promoted the production of this therapeutic cytokine, TGF-β1. Although the induction of other regulators is not excluded, the TGF-β1-inducing activity is postulated to be a predominant mode of action of SEK-1005 in causing acceleration of wound healing because the elevated levels of TGF-β1 in wound exudate were closely correlated with the wound-repairing effects in the animals treated with SEK-1005. However, other experiments, for instance to relate the synthesis of TGF-β1 to cellularity or the accumulation of specific cell types, e.g. macrophages or fibroblasts, are required to define better the mechanism of action of SEK-1005.

There have been several reports on the production of TGF-β1 by platelets, macrophages and fibroblasts (Assoian et al., 1983, 1987; O'Kane and Ferguson, 1997). Although the mechanism by which SEK-1005 enhances TGF-β1 production is far from being fully understood, the compound seems to stimulate platelets and/or macrophages (Roberts, 1995), as it was demonstrated to increase the secretion of TGF-β1 6 h after the wound was made in a preliminary study.

It can be appreciated from the above that SEK-1005 is a new type of accelerator of wound healing and has potent TGF-β1 inducing activity. A growth factor-inducer, such as SEK-1005, appears to be more effective than exogenous growth factor during the orderly progression of wound healing. Additionally, SEK-1005 has another interesting activity for wound healing. It has antibacterial activity (minimum inhibitory concentration: 10 ng/ml)(Fujiwara et al., 1999) against Gram-positive bacteria including *Staphylococcus aureus*, infection with which is reported to delay wound healing (Okumura et al., 1996b). Further experiments are in progress with a view to predicting clinical efficacy.

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