Sensory Peptides as Neuromodulators of Wound Healing in Aged Rats

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Inflammation is essentially a protective response that initiates the process of tissue repair (Holzer, 1988; Otsuka and Yoshioka, 1993). A major part of the normal inflammatory response in tissue is dependent upon intact unmyelinated primary afferent sensory innervation (Kjatansson et al., 1987; Maggi et al., 1987a; Parkhouse and LeQuenese, 1988a, 1988b; Holzer, 1991). These particular afferents are termed capsaicin-sensitive primary afferents because they are selectively destroyed by the neurotoxin capsaicin, the pungent extract of red peppers of the genus Capsicum. Thus animals pretreated as neonates with the neurotoxin capsaicin have permanent loss of primary afferent unmyelinated nerves, depletion of their sensory neuropeptides, and show reduced inflammatory responses, poor wound healing, and diminished skin integrity (Kjatansson et al., 1987). There is additional evidence that capsaicin-sensitive sensory fibers exert trophic functions to maintain tissue integrity. Capsaicin-treated animals exhibit corneal opacities (Buck et al., 1983; Fujita et al., 1984), skin lesions (Buck et al., 1983; Maggi et al., 1987a), delayed fur regrowth (Maggi et al., 1987a), a decreased survival of skin flap (Kjatansson et al., 1987), and an increased susceptibility to develop gastrointestinal ulcer (Maggi et al., 1987b).

Unmyelinated afferent nerves with polymodal nociceptors mediate early components of the neurogenic inflammatory response — namely early neutrophil migration (Eglezos et al., 1989, 1991), plasma extravasation (Khalil and Helme, 1989), and local vasodilatation (Khalil and Helme, 1990) — and modulate cellular components of the immune response (Payan et al., 1986; Eglezos et al., 1991). These effects are mediated by neuropeptides contained in peripheral terminals of primary afferent nerves.

With aging there is also a decrease in the capacity of individuals to maintain appropriate inflammatory and repair processes. For example, both animal (Khalil, 1993) and human (Eaglstein, 1989) studies have suggested that wound healing, as measured by wound closure time, is impaired with age. We have recently provided evidence in aged rats for changes in sensory nerve function at both pre- and postterminal levels (Khalil et al., 1994). We have also documented a decrease in peptide synthesis, content, and release in old rats and an overall decrease in neurogenic inflammation with antidiromic nerve stimulation, and raised the possibility that these changes may have important implications for wound healing and repair mechanisms.

The overall aim of this study was to investigate the concept that the decline in sensory nerve function with age contributes significantly to poor healing qualities of tissues in aged animals. We examined the role of sensory nerves and the effect of aging on the healing of a thermal burn induced on the interscapular region of the rat. In the second part of our study, we made use of our previous findings, that tachykinin receptors in old rats are upregulated (as a consequence of a preterminal defect in peptide release mechanisms of sensory nerves) to investigate the role of substance P (SP) and calcitonin gene-related peptide (CGRP) in modulating wound healing in old rats, with specific emphasis on the importance of the modulatory interactions of these peptides.

Methods

Animals. — Young control and capsaicin-pretreated rats (3 months old) and aged (24 months old) Sprague-Dawley male rats were used. For capsaicin pretreatment, neonatal
rats were given a single subcutaneous dose of 50 mg/kg of capsaicin on 2-4 days of life. The purpose of this treatment was to permanently destroy small diameter sensory nerves. Capsaicin was prepared in normal saline containing 10% Tween-80 and 10% alcohol. The effectiveness of neonatal capsaicin pretreatment was tested before each experiment using the eye-wiping response to topical application of capsaicin (0.1% in alcohol) to the cornea. Denervation with capsaicin was later confirmed by assaying SP levels in the skin. Data are only included if capsaicin pretreatment results in >80% depletion in SP levels.

**Thermal burn.** — The fur on the interscapular region was removed with animal clippers and a cosmetic depilatory agent. At least 24 h after this treatment, rats were anesthetized with sodium pentobarbital (65 mg/kg i.p.) and a thermal burn was induced in the treated region by using a CO\(_2\) laser (four consecutive stimulation periods each at 25 W power, 0.5-sec duration, and spot diameter of 10 mm). This technique delivers standard energy levels over a given area of skin and therefore gives a similar and reproducible thermal injury. This injury results in a large circular wound area (2 cm\(^2\)). The wound area increases to 2.5-3 cm\(^2\) by day 2 due to progressive loss of the microcirculation because thermal injury damages capillary endothelial cells. This shape and size of wound allow for healing primarily by contraction and re-epithelialization over approximately 3 weeks in old rats. It is also known that round wounds do tend to contract more uniformly, rarely deform into two or more wounds, and are associated with less measurement error (Richey et al., 1989).

**Measurement of healing.** — The area of burn was traced daily for the first 6 days and every 48 h thereafter and measured with a digital planimeter. Measurements were made by an observer who was unaware of the treatment status of the animals. Wounds were left uncovered and observed daily. Any lightly adherent eschar was removed with forceps. Although scab formation was minimal, when it occurred, the scab was gently removed. This was done to keep all wounds comparable as previous studies by Snowden et al. (1982) showed that scab formation induces a transient decrease in the rate of wound contraction. It was also easier to accurately measure open wound area without the overlying scab. The healing endpoint was determined as the time when full wound contraction had occurred. It should be noted that although wound contraction is one only parameter of wound healing, it accounts for large portions of wound closure in full-thickness wounds.

The modulatory interaction effect between sensory peptides by using the blister model. — Young and old rats (3 and 24 months old, respectively) were anesthetized with pentobarbital sodium (60 mg/kg i.p.). Further doses of 15 mg/kg anesthetic were administered to ensure that rats stayed under anesthesia. Body temperature of the rats was maintained at 37°C.

A blister was induced in the right hind footpad of the rat by applying a suction pressure of -40 kPa for approximately 30 min by using a metal suction cap heated to 40°C (Khalil and Helme, 1989, 1990; Khalil et al., 1994). The surface epithelium was removed and the rat foot secured in a perspex chamber with inlet and outlet ports. Ringer’s solution was perfused over the blister base at 4 ml/h by means of a peristaltic pump (Microperpex S; LKB, Sweden). Relative blood flow was monitored over time by a laser Doppler flowmeter (Periflux, PF2B; Perimed, Sweden) via a probe placed in a central port immediately above the blister base; relative blood flux (volts) was continuously monitored on a chart recorder. At the end of the experiment, animals were killed by barbiturate overdose. An initial equilibration period of 30 min was allowed, during which time a stable baseline was achieved. In control experiments with young and old rats, either SP or CGRP (both at 1 μM) were perfused for 30 min or 10 min, respectively, over the blister base. To examine possible interaction modulatory effects between CGRP and SP, in a separate set of experiments, 1 μM of CGRP was perfused for 10 min followed by a 10-min poststimulation period with Ringer’s solution; then SP (at 0.1, 1 or 10 μM) was perfused for 30 min. Furthermore, the involvement of SP receptor in the modulatory effects exerted by SP on the vasodilator response to CGRP was examined in another group of rats. Subsequent to perfusion of CGRP and Ringer’s solution, an effective tachykinin antagonist (Spantide II) (Folkers et al., 1990; Hakanson et al., 1991; Xu et al., 1991) was perfused together with SP (all at 1 μM).

**Drug injection.** — Peptide (200 μl) was injected intradermally at four sites 1 cm distant to the burn edge twice daily (6-8 h apart) for 5 days after thermal injury. Six groups of 2-year-old rats were used: group 1, saline; group 2, SP; group 3, CGRP; group 4, SP and CGRP; group 5, CGRP after SP antagonist, and group 6, CGRP (after SP antagonist) and SP. In all groups except group 6, the same treatment was used twice daily. However, group 6 received Spantide II as part of the initial treatment with CGRP and 6 h later an injection of SP alone. The rationale behind using the SP antagonist before CGRP injection was to allow the latter to maintain its vasodilator response. All peptides were used at 10 μM. Five days of treatment were chosen because our previous studies showed that old rats and capsaicin-pretreated rats healed significantly more slowly than adult rats, with the most significant difference in wound size occurring in the first 6 days. Both groups showed a lag period of around 4 days before wound contraction began.

**Drugs.** — Nembutal (pentobarbital sodium) was from Boehringer Ingelheim Ltd., Australia. SP and CGRP were from Auspep, Australia, while the SP antagonist (Spantide II) was from Peninsula Laboratories.

**Statistics and expression of data.** — All results are expressed as mean ± SEM (n = 7-8 in each group). Statistical analyses were performed using a two-way (group × days) repeated measures analysis of variance (ANOVA) followed by post hoc Duncan’s pairwise comparisons. A p-value < .05 was considered significant.
RESULTS

Wound healing in young, old, and capsaicin-pretreated rats. — The time required to complete wound closure was monitored in three groups of rats, young (3 months; control), capsaicin-pretreated rats at 3 months of age, and 2-year-old rats. Young control rats showed an early increase in wound size that reached a maximum by day 2 followed by healing, manifested as a gradual reduction in wound size. Complete wound closure was achieved at 14.9 ± 0.2 days (see Figure 1). Old rats and young capsaicin-pretreated rats showed a different profile of healing compared with young controls. In both groups, the wound reached maximum size by day 2 (similar to young control rats); however, there was a lag phase before an actual reduction in wound size observed by day 5. Complete wound closure occurred at 21.1 ± 0.3 and 21.3 ± 0.6 days for old and capsaicin-pretreated rats, respectively (Figure 1).

Effect of SP or CGRP on skin microvasculature in young and old rats. — Perfusion of 1 μM SP over the blister base induced a vasodilatation (VD) response that is characterized by tachyphylaxis within 15 min in young rats. This vasodilator response was significantly enhanced in old rats, while the subsequent tachyphylaxis was less prominent (Figure 2A). The area under the response curve for 30-min perfusion in young rats was 29.3 ± 5.3 cm² as opposed to 55.9 ± 6.8 cm² in old rats.

Perfusion of 1 μM CGRP for 10 min over the blister base induced an immediate VD response in young rats that reached maximum within 1–2 min and was maintained for more than an hour after cessation of stimulation. A similar response was obtained in old rats; however, the poststimulation period showed a greater amplitude of response compared to young rats (Figure 2B). The area under the response curve for 10-min perfusion followed by 10-min poststimulation with Ringer’s solution in young rats was 45.7 ± 4.9 cm² compared with 64.8 ± 7.2 cm² in old rats.

Modulation of the vascular response to CGRP by SP. — In these experiments, 1 μM CGRP induced a vasodilator response that was maintained during the poststimulation period with Ringer’s solution. Subsequent SP perfusion at 1 μM terminated the existing vasodilator response to CGRP (Figure 3A). The modulatory effects exerted by increasing concentrations of SP (0.1, 1, and 10 μM) on the vasodilator response to 1 μM CGRP were dose dependent (Figure 3B). The higher the concentration of SP, the quicker it terminates the vasodilator response to CGRP. The possibility that this modulatory effect of SP on the vasodilator response to CGRP is mediated via an action on NK-1 receptor was examined by using an NK-1 receptor antagonist (Spantide II). When this antagonist was concomitantly perfused with SP (all at 1 μM), it was able to prevent SP from terminating the vasodilator effect of CGRP (Figure 3A).

Effect of SP and/or CGRP treatment on wound healing in old rats. — The effect of two daily i.d. injections of 200 μl SP or CGRP (both at 10 μM) on wound healing in old rats is shown in Figure 4. Old rats treated with SP showed less enlargement of the wound compared to saline-treated con-

Figure 1. Wound healing over time in young, old, and capsaicin-pretreated rats. Complete wound closure was achieved at 14.9 ± 0.2, 21.1 ± 0.3, and 21.3 ± 0.6 days, respectively. From day 4 onward, post hoc analysis showed that wound size in the young control group was significantly less than the other two groups.

Figure 2. Both A and B provide tracings of a typical record of relative blood flow monitored by using a laser Doppler flowmeter, showing the vasodilator response of the skin microvasculature to SP and CGRP, both at 1 μM, respectively. In contrast to the maintained response to CGRP in both young and old rats, the response to SP was characterized by tachyphylaxis, which was more prominent in young rats.
SENSORY NERVE PEPTIDES AND WOUND HEALING

Figure 3. (A) Tracing of a typical record of relative blood flow monitored by using a laser Doppler flowmeter, showing that subsequent perfusion of SP can terminate an existing vasodilator response to CGRP (both peptides used at 1 μM). The figure also shows that the modulatory effect of SP can be prevented by concomitant perfusion of the tachykinin antagonist (Spantide II) at 1 μM. (B) The modulatory effect exerted by SP on the vasodilator response to CGRP is dose dependent. The higher the concentration of SP, the quicker it terminates the vasodilator response to CGRP.

Figure 4. Effect of two daily i.d. injections of 200 μl of CGRP or SP (10 μM each) or combined treatment with the two peptides on the wound size and healing in old rats. Old rats treated with SP showed an accelerated wound closure that was significantly different from control by day 4 onward, with complete wound closure occurring at 16.3 ± 0.4 days. Treatment with CGRP increased wound size compared with controls during the first 5 days, with this difference reaching statistical significance on day 4. A significant reduction in wound size in response to CGRP was determined by day 8 onward, with complete wound closure achieved by 17.1 ± 0.4 days. The combined treatment resulted in complete wound closure at 17.3 ± 0.6 days compared with 16.3 ± 0.4 and 17.1 ± 0.4 days for SP or CGRP, respectively.

Effect of a combined treatment of a SP antagonist with CGRP on wound healing. — In an attempt to prolong the vasodilator effect of CGRP, we preceded the CGRP treatment (10 μM) with an injection of the tachykinin antagonist (Spantide II) (10 μM). This treatment significantly reduced the time to complete wound closure, with healing achieved at 14.9 ± 0.3 days compared with 17.1 ± 0.4 days using CGRP alone (Figure 5). This combined treatment, however, still had the disadvantage of an increased wound size during the first 4 days, which was significantly greater than control. A significant healing effect was observed by day 6.

Effect of a combined treatment of a SP antagonist and CGRP followed by a SP treatment on wound healing. — In an attempt to overcome the early phase of increased wound size and at the same time accelerate wound healing, we combined the prolonged CGRP treatment (achieved by preceding the injection of CGRP with an injection of the SP antagonist (see above)) with a second injection of SP 6 h later. This protocol resulted in the most rapid healing, with complete wound closure at 11.3 ± 0.3 days compared to 21.1 ± 0.9 days in saline-treated old control rats (Figure 6).
preceeded with an injection of the tachykinin antagonist (Spantide II) (all at 10 μM). This treatment resulted in reduction of time required to complete wound closure compared with saline-treated controls and the CGRP-treated group. This treatment protocol increased wound size during the initial phase; however, a significant healing effect was observed by day 6, with complete wound closure achieved at 14.9 ± 0.3 days.

DISCUSSION

Sensory nerves have been known to exert a trophic action on human skin for over 50 years (Holzer, 1988). A number of sensory neuropeptides are present in unmyelinated sensory nerve fibers of mammalian skin and may mediate this trophic action. Animals pretreated as neonates with the selective sensory neurotoxin, capsaicin, show permanent sensory and functional deficits which involve both the afferent and local effector functions of sensory neurons (Holzer, 1991). These animals also show poor wound healing and skin integrity (Kjatansson et al., 1987).

Healing of injured tissues requires adequate organizational and functional reactions of the primary afferent sensory nerves and the microcirculatory system. Insufficient or improper coordination of these processes may be expected to predispose to trophic disorders of the skin. Support for this conjecture comes from clinical observations that sensory neuropathies may be associated with persistent skin ulcers (Bockers et al., 1989) and connective tissue diseases (Hagen et al., 1990). Experimentally, ablation of capsaicin-sensitive afferent neurones reduces the survival of a musculocutaneous flap (Kjatansson et al., 1987) and can lead to appearance of persistent skin wounds (Maggi et al., 1987a), aggravation of acid-induced skin lesions (Maggi et al., 1987b), and formation of keratitis-like lesions in the cornea of small rodents.

A role of sensory afferent neurones in wound healing can also be envisaged from the ability of SP, neurokinin A (NKA), and CGRP to stimulate the proliferation of endothelial cells (Haegerstrand et al., 1990), arterial smooth muscle cells (Nilsson et al., 1985; Payan et al., 1986), and skin fibroblasts (Nilsson et al., 1985). These actions are likely to play a role in the angiogenesis of healing tissue, and SP is in fact able to induce neovascularization of the vascular rabbit cornea (Ziche et al., 1990b). SP is also known to cause neurite outgrowth in embryonic chick dorsal root ganglia (Narumi and Fujita, 1978) and cultured neuroblastoma cells (Narumi and Maki, 1978). Furthermore, SP stimulates DNA synthesis in cultured arterial smooth muscle cells and human skin fibroblasts, and this effect is inhibited by a tachykinin antagonist (Nilsson et al., 1985). All these effects may be involved in wound healing and trophic functions of C-afferent fibers.

Dysfunction of sensory afferent neurones with age is likely to have a bearing on the pathophysiology of the skin. Both animal (Khalil, 1993) and human studies (Eaglstein, 1989) show that wound healing is impaired with age. Wound contraction begins later and proceeds more slowly. The major reason for delayed healing appears to be a prolonged lag phase between injury and the start of wound contraction.

Wound healing is a complex process, and several models have been used to study distinct aspects of the overall event. Full-thickness wounds, including our thermal burn wound, are known to heal by contraction as well as re-epithelialization. It should be noted that full-thickness burn wounds in humans could be complicated by excessive connective tissue remodeling and permanent scar formation. In this study, wound size and time to complete wound closure were measured following a standard thermal scar formation. We used the change in wound size, as monitored by the open wound area, as the outcome measure of wound healing. We believe that our measurements documenting the change in surface wound area over time could be representative of two aspects of wound healing — namely, contraction and re-epithelialization. However, because quantitative histological and immunohistochemical techniques were not applied in this study, the authors concede that assessment of wound size as reported here might not exactly reflect the true extent of wound contraction or re-epithelialization.

In the first part of the study, the importance of sensory nerves and the effect of aging on wound healing using our thermal wound model were examined in capsaicin-pretreated rats and in 2-year-old rats, respectively.

Our results demonstrate that both old and capsaicin-pretreated groups showed delayed wound contraction and
sensory nerve receptors around the wound that might act to increase wound size from day 4 onward together with a significant reduction in wound size during this early phase, which argues in favor of the proposition that the increase in wound size by CGRP could be related to its potent vasodilator activity. Despite a similar increase in wound size in response to CGRP in the presence of a SP antagonist, it should be mentioned that this treatment protocol resulted in a faster time to complete wound closure (14.9 ± 0.3 days) compared with 17.1 ± 0.4 days in response to treatment with CGRP alone.

The ability of CGRP to accelerate wound healing despite the early increase in wound size could be attributed to its known trophic effects exerted by this peptide, including its ability to influence innervation either directly as suggested by Karanath et al. (1990) or indirectly through its angiogenic properties (Haegerstrand et al., 1990). In support of this argument, previous studies by Kjatansson and Dalsgaard (1987) showed that the ability of CGRP to increase survival of a musculocutaneous flap was an effect independent of its vasodilator activity. It should be noted that part of the initial increase in wound size in the presence of CGRP and the SP antagonist could be related to the ability of the antagonist to block the healing effect of the endogenously released SP.

SP is also capable of exerting similar trophic functions and may stimulate DNA synthesis in arterial smooth muscle cells and skin fibroblasts (Nilsson et al., 1985) and is able to promote the growth of capillary vessels (Ziche et al., 1990b). These effects, combined with a limited vasodilator activity compared to CGRP, might have allowed SP to exert a potent significant healing effect earlier than CGRP by overcoming the early delayed phase in wound contraction.

In an attempt to optimize the effect of sensory peptides on wound healing, we then combined the two treatments that induced the most significant healing effect — namely the prolonged CGRP effect (achieved by preceding the injection of CGRP with an injection of the SP antagonist) followed by a second injection of SP 6 h later in an attempt to overcome the early delay phase. This treatment protocol resulted in an early healing effect, characterized by a significant reduction in wound size from day 4 onward together with a significant reduction in time to complete wound closure, an improvement of 46% over saline-treated controls.

Therefore, our data provide direct evidence for the ability of sensory peptides to accelerate wound healing and indirect evidence to suggest a possible role for sensory peptides in modulating wound contraction and re-epithelialization. This latter proposition is supported by previous studies by
Watcher and Wheeland (1989) provided evidence that topical application of capsaicin accelerated wound contraction and re-epithelialization of full-thickness wounds in swine. The authors suggested that the effects of capsaicin might be mediated via the release of SP from sensory nerves.

Our current findings demonstrating the ability of sensory peptides to accelerate tissue healing should not undermine the important role of locally acting growth factors in the regulation of wound repair. It is well known that endogenous growth factors are released at the wound site and play a necessary part of the natural wound healing process (for a review see Martin et al., 1992). Studies by Nanney (1990) and Stoscheck et al. (1992) provided evidence that epidermal growth factor (EGF) stimulates the rapid epithelialization of skin wounds with an increase in EGF-receptor expression in the epidermis during the proliferation and migration of epidermal keratinocytes. Furthermore, Seyfer et al. (1990) demonstrated that cell-bound EGF is processed into multiple forms and that the mechanisms of processing EGF and the time course of these events may explain the lag phase of wound healing. Recent studies by Tsuboi et al. (1995) demonstrated that coadministration of insulin-like growth factor (IGF) and IGF-binding protein 1 stimulates wound healing of full-thickness wounds of diabetic mice and normal rabbits. In another study by Robson et al. (1994), the authors provided evidence that topical application of granulocyte-macrophage colony stimulating factor inhibits the retardation of wound closure produced by bacterial contamination.

It is certainly true that sensory peptides and growth factors could have an overlapping range of actions that are essential for tissue repair, including a role in angiogenesis, cell proliferation and migration, synthesis of extracellular matrix, wound contraction, and re-epithelialization. In support of this notion, a recent immunohistochemical study by Manek et al. (1994) using ischaemic skin flaps demonstrated an increase in CGRP-immunoreactive nerve fibers and raised the possibility that CGRP may be able to influence innervation either directly, as suggested by Karanath et al. (1990), or indirectly through its angiogenic properties (Haegestrand et al., 1990). SP is also known to exert a powerful mitogenic action on different cell types which, like fibroblasts (Nilsson et al., 1985; Ziche et al., 1990a), endothelial cells (Ziche et al., 1990b), and macrophages (Brunelleschi et al., 1990), are involved in tissue repair. In addition to the ability of SP to stimulate the proliferative responses of smooth muscle cells and skin fibroblasts (Nilsson et al., 1985), it also stimulates cytokine production by mononuclear cells and is capable of stimulating the production of interleukin 1, transforming growth factor α, and EGF by eosinophils and macrophages (Lotz et al., 1988). Therefore it is reasonable to suggest that the ability of sensory peptides to modulate wound healing could be attributed partly to their ability to induce the production of certain growth factors by inflammatory cells.

In summary, we have examined the role of sensory nerves and the effect of aging on wound healing of a thermal burn induced on the interscapular region of the rat. We have provided evidence to support the notion that sensory nerves are important for the initiation of healing and tissue repair and showed that wound healing is significantly delayed with age. The results also provide evidence that sensory peptides accelerate wound healing in aged rats and that the interaction between these peptides can influence their ability to modulate the healing process. This study will bring valuable advances in the clinical management of impaired wound healing with age and raises the possibility of the future use of similar treatment protocols to accelerate wound healing in aged humans.

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