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Nerve growth factor: Acceleration of the rate of wound healing in mice

(submandibular glands/plasminogen activation/serine proteases)

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ABSTRACT **Earlier studies have shown that removal of the submandibular glands of mice retards the rate of contraction of experimentally induced wounds and that communal licking of wounds accelerates contraction in intact animals [Hutson, J. M., Niall, M., Evans, D. & Fowler, R. (1979) *Nature (London)* 279, 793–795]. In the light of the observation that nerve growth factor (NGF) is secreted in high concentrations in mouse saliva, we have studied the effect of topically applied high molecular weight nerve growth factor (HMW-NGF) upon the rate of wound contraction in sialoadenectomized animals. Results show that HMW-NGF significantly accelerates the rate of wound contraction and that this phenomenon is probably dependent upon the enzymic activity of the protein. Neither diisopropyl fluorophosphate-inactivated NGF nor 2.5S NGF [isolated according to Bocchini, V. & Angeletti, P. U. (1969) *Proc. Natl. Acad. Sci. USA* 64, 787–794] displays this biological activity. Thus, it may be that one of the physiological roles of NGF in saliva is to promote wound healing by the licking process.**

Recent studies have shown that the predominant form of male mouse submandibular gland nerve growth factor (NGF) is a 116,000 molecular weight protein (1) that is secreted in high concentrations in saliva (2–4). This high molecular weight nerve growth factor (HMW-NGF) also exhibits some unusual enzymic properties. For example, it can hydrolyze certain N-substituted lysine and arginine methyl esters [e.g., α-*N*-*p*-tosyl-L-arginine methyl ester (TAME)] (5, 6), and it also has the capacity to activate plasminogen (6). The proteolytic activity of HMW-NGF can be inhibited by reaction with diisopropyl fluorophosphate (iPr_2P-F), and therefore the protein is a member of the general class of serine proteases (6).

As isolated and purified from the submandibular gland, HMW-NGF exists as a catalytically inactive zymogen, and it can be subsequently converted to active enzyme by autocatalytic activation (7). For example, upon dilution of the zymogen from high to low protein concentrations, or by treatment of the protein with metal ion chelating reagents, autocatalytic activation ensues with subsequent appearance of full enzyme activity. This activation reaction has now been shown to be under the control of 1 mol of tightly bound Zn(II) per mol of protein. Removal of this ion by dilution or chelation initiates autocatalysis (8).

Several years ago, Levi-Montalcini and Angeletti (9) observed that experimentally induced mouse granulation tissue displayed NGF-like activity. Subsequently, Young *et al.* (10) observed that primary chick fibroblasts have the capacity to secrete NGF in culture. Because fibroblasts are a prominent feature of granulation tissue, we speculated that NGF might play some biological role in the process of wound healing.

The preceding observations are pertinent to recent studies of Hutson, *et al.* (11) on the effect of salivary glands upon wound contraction in mice. These authors found that the mouse submandibular and sublingual glands contain a substance(s) that is applied to wounds primarily by the licking process and that accelerates early wound contraction. Because NGF is present in high concentrations in mouse saliva and seems to be a natural component of granulation tissue, we examined the effect of purified submandibular gland NGF upon contraction of experimentally induced wounds. Results indicate that when pure mouse HMW-NGF is applied topically to superficial skin wounds of sialoadenectomized mice, the rate of wound contraction is significantly and consistently accelerated. This effect is not displayed by 2.5S NGF, iPr_2P-F-inactivated NGF, trypsin, or urokinase.

MATERIALS AND METHODS

Reagents. NGF zymogen (7), 2.5S NGF (12), and iPr_2P-F-inactivated NGF (6, 8) were prepared as described. All preparations were homogeneous in electrophoresis, chromatography and the ultracentrifuge. Enzyme assays with TAME as substrate were used to insure inactivation of the proteolytic activity of HMW-NGF by iPr_2P-F (7). iPr_2P-F was obtained from Pfaltz and Bauer (Stamford, CT); TAME was obtained from Sigma; and twice-recrystallized trypsin was obtained from Worthington. Human urokinase was a gift of N. S. Orenstein (Massachusetts General Hospital). All proteins were dissolved to the desired concentration in 0.1 M potassium phosphate (pH 7.0).

Animals. Forty-day-old Swiss strain male mice (26–28 g body weight) were obtained from Charles River Breeding Laboratories. Submandibular glands of some animals were removed under ether anesthesia through a midline incision; other animals underwent a sham operation that involved full mobilization of the glands and subsequent incision closure with 3-0 silk.

Wounds were made under anesthesia immediately after either sialoadenectomy or sham operation. A rubber stamp of 1 cm^2 stained with India ink was applied to a shaved sacral area on the back of each animal and full-thickness skin (including the panniculus carnosus muscle) was excised. Photography at fixed focal length was used to measure wound areas on alternate days as follows. A glass slide with ruler marker was pressed onto each wound so that a flat surface was presented for photography. Color transparencies of the wounds were projected onto uniform weight paper, and tracings of wound areas were cut

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Abbreviations: NGF, nerve growth factor; HMW-NGF, high molecular weight nerve growth factor, purified as in ref. 1; 2.5S NGF, NGF isolated as in ref. 12; iPr_2P-F, diisopropyl fluorophosphate; TAME, α-*N*-*p*-tosyl-L-arginine methyl ester.

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out and weighed. Student's *t* test was used for analysis of the data. The animals were housed in wire mesh cages under uncrowded conditions and were fed laboratory Purina chow and water *ad libitum*.

To explore the effect of sialoadenectomy versus sham operation, we divided the animals into three groups. Twenty-five sham-operated animals were housed together in one cage. A second group of 25 sialoadenectomized animals was placed in a separate cage, and a group of 18 mice, half of which were sham-operated and half sialoadenectomized, was placed in a third cage.

To study the effects of topically applied reagents, we sialoadenectomized a total of 120 mice on two separate occasions and divided them into six groups, separately caged. These six groups of animals were treated topically with HMW-NGF, 0.1 mM phosphate buffer, iPr_2P-F-inactivated NGF, 2.5S NGF, trypsin, and urokinase, respectively. The treatment schedule for each reagent consisted of application to the wound area immediately after injury (zero time) and three additional applications at 12-hr intervals (total treatment was restricted to a 36-hr period).

RESULTS AND DISCUSSION

To establish baseline measurements for the effect of sialoadenectomy upon wound contraction in our system, we have repeated some of the studies of Hutson *et al.* (11). Fig. 1 presents the percentage of the initial wound areas as a function of time after injury for both sham-operated and sialoadenectomized animals. In agreement with the results of Hutson *et al.* (11), there was a highly significant difference ($P < 0.001$) in wound areas between animals with and without submandibular glands up to 8 days after injury. The data in Fig. 1 were obtained with groups of animals (sham-operated and operated) that were housed separately. When animals from the two groups were housed together in the same cage, the rates of wound closure were not significantly different at any time point from those of mice with intact submandibular glands. This finding is in complete agreement with the results of Hutson *et al.* (11), and it indicates that the licking process is somehow intimately involved in accelerating the rate of wound healing.

To evaluate the effect of HMW-NGF upon wound contraction, 50 μl of a solution (110 μg/ml) of the protein in potassium phosphate buffer was applied topically to wound areas of sialoadenectomized animals immediately after injury, with three additional applications at 12-hr intervals. This concentration of HMW-NGF was chosen because it is representative of that present in normal male mouse saliva (3). As shown in Fig. 1, the degrees of wound contraction displayed by mice treated with NGF during a 36-hr period were similar to those of sham-operated animals up to day 4, and they differed markedly from wound areas of control sialoadenectomized animals ($P < 0.001$). Even at day 6, NGF-treated wounds differed significantly from those of untreated or buffer-treated sialoadenectomized animals ($P < 0.01$). By day 8 and beyond, NGF-treated wound areas were comparable to those of sialoadenectomized mice. Together these results indicate that NGF significantly accelerates the rate of wound contraction in animals without submandibular glands.

The NGF zymogen, once activated by autocatalysis, can hydrolyze certain lysine and arginine esters. It also contains as

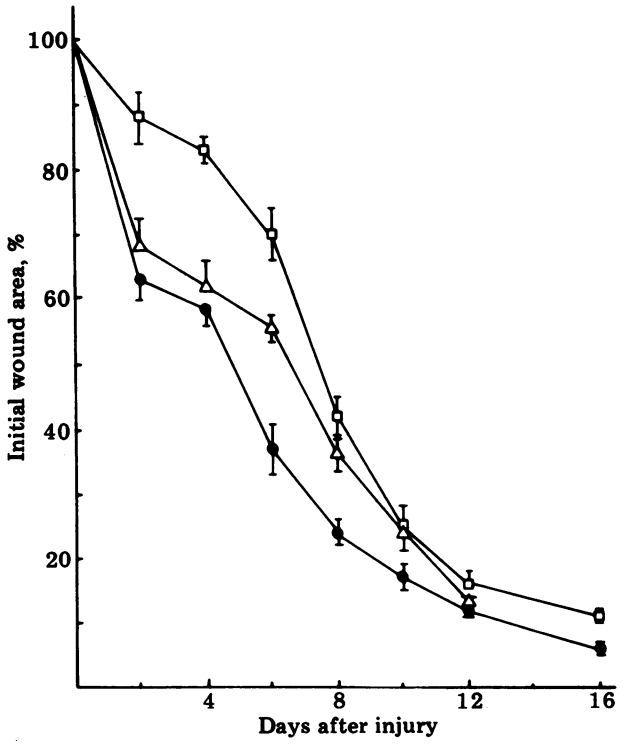

FIG. 1. Effects of sialoadenectomy and of NGF upon rates of wound contraction. Each time point represents data from 25 animals. □, Sialoadenectomized mice housed together in the same cage; ●, sham-operated mice housed in one cage; △, HMW-NGF-treated mice housed in one cage [50 μl of HMW-NGF (110 μg/ml) was applied topically to the wounds of sialoadenectomized animals immediately after injury, with three additional applications at 12-hr intervals]. Values are means ± SEM.

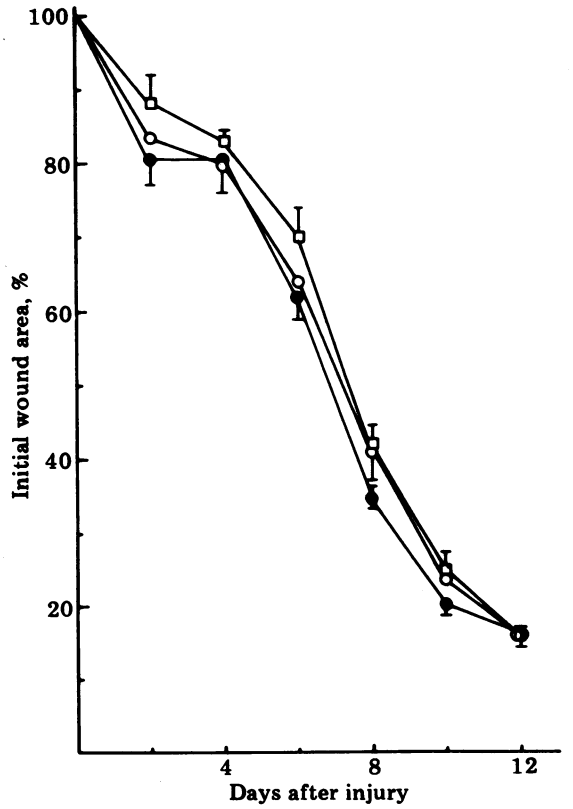

FIG. 2. Effects of 2.5S NGF and iPr_2P-F-inactivated NGF upon rates of wound contraction. Each time point represents data from 18–30 animals. □, Buffer-treated sialoadenectomized mice; ○, sialoadenectomized mice treated with 50 μl of 2.5S NGF (27 μg/ml) at the times described in the legend to Fig. 1; ●, sialoadenectomized mice treated with 50 μl of HMW-NGF (110 μg/ml) that had been inactivated by iPr_2P-F. Values are means ± SEM.

part of its subunit structure the nerve growth-promoting 2.5S NGF. To evaluate the role of the enzymic activity of HMW-NGF and of its nerve growth-promoting subunit upon rates of wound contraction, solutions of iPr_2P-F-inactivated NGF and of 2.5S NGF were applied topically to wounds in concentrations equimolar to those of the native HMW-NGF in Fig. 1. Neither of these proteins displayed any significant effect (Fig. 2) upon the extent of wound contraction when compared to buffer-treated sialoadenectomized animals (4 days after injury, $P < 0.01$ for HMW-NGF versus the iPr_2P-F-inactivated protein and $P < 0.001$ for HMW-NGF versus 2.5S NGF). These findings suggest that the enzyme activity of the HMW-NGF molecule may be required for acceleration of contraction and that its nerve growth-promoting subunit is inactive in this experimental system. They further suggest that perhaps the plasminogen-activating property of HMW-NGF is responsible for the observed results. However, this seems unlikely since we have been consistently unable to detect any effect of topically applied urokinase or trypsin (in concentrations equimolar to that of the HMW-NGF in Fig. 1) upon rates of wound contraction in sialoadenectomized mice.

Wound healing is a complicated and poorly understood process that involves participation of several different cell types. In agreement with the findings of Hutson *et al.* (11), the results presented above indicate that mutual licking is involved in promoting wound healing and that the submandibular glands of mice play a role in this process. Moreover, when purified salivary gland HMW-NGF is applied to animals without submandibular glands, the rate of wound contraction is markedly accelerated. The combined observations (*i*) that mouse granulation tissue contains an NGF-like activity (9), (*ii*) that fibroblasts can secrete NGF (10), and (*iii*) that NGF is present in high concentrations in mouse saliva (2–4) suggest that secretion of NGF by cells prominent in granulation tissue may be important in the wound-healing process. The results presented here suggest that promotion of wound healing may be one of the physiological functions of NGF in saliva.

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1. Young, M., Saide, J. D., Murphy, R. A. & Blanchard, M. H. (1978) *Biochemistry* **17**, 1490–1498.
2. Wallace, L. J. & Partlow, L. M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4210–4214.
3. Murphy, R. A., Saide, J. D., Blanchard, M. H. & Young, M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2330–2333.
4. Murphy, R. A., Saide, J. D., Blanchard, M. H. & Young, M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2672–2676.
5. Greene, L. A., Shooter, E. M. & Varon, S. (1968) *Proc. Natl. Acad. Sci. USA* **60**, 1383–1388.
6. Orenstein, N. S., Dvorak, H. F., Blanchard, M. H. & Young, M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5497–5500.
7. Young, M. (1979) *Biochemistry* **18**, 3050–3055.
8. Young, M. & Koroly, M. J. (1980) *Biochemistry*, in press.
9. Levi-Montalcini, R. & Angeletti, P. U. (1961) in *Regional Neurochemistry*, ed. Kety, S. S. & Elkes, J. (Pergamon, Oxford), p. 362.
10. Young, M., Oger, J., Blanchard, M. H., Asdourian, H., Amos, H. & Arnason, B. G. W. (1975) *Science* **187**, 361–362.
11. Hutson, J. M., Niall, M., Evans, D. & Fowler, R. (1979) *Nature (London)* **279**, 793–795.
12. Bocchini, V. & Angeletti, P. U. (1969) *Proc. Natl. Acad. Sci. USA* **64**, 787–794.