Enhancement of Wound Healing by the Alkaloid Taspine Defining Mechanism of Action (43567)

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Abstract. **Taspine (mol wt 369,000) is an alkaloid extracted from trees of Croton (family Euphorbiaceae) of the western Amazon region that has been used by natives and others as a vulnerary agent. Taspine was purified from tree sap to test its healing properties using different topical concentrations in the paired rat surgical incision model. Wound tensile strength and histology were evaluated. Samples treated with 250 μg, but not those treated with 50 μg or 10 μg, had significant higher values for MBS than paired controls (26%, $P < 0.005$, and 30%, $P < 0.001$, by Days 5 and 7, respectively). Taspine did not modify MBS at Day 12. Sample treated with 250 μg had significantly greater mononuclear cellular infiltration at Days 5 and 7 but not at Day 12. To better understand the effect of taspine as an enhancer of wound healing, we conducted *in vitro* studies in cell cultures. Taspine stimulated chemotaxis for fibroblasts. Taspine did not have an effect on specific assays for macrophage chemotaxis, neutrophil activation, fibroblast proliferation, or matrix assembly. Taken together, the data suggest that taspine promotes early phases of wound healing in a dose-dependent manner with no substantial modification thereafter. Its mechanism of action is probably related to its chemotactic properties on fibroblasts and is not mediated by changes in extracellular matrix.**

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The availability of substances capable of stimulating the process of wound repair is limited and potentially costly. Recently, we successfully reported acceleration of wound healing by administration of growth factors (1–7), and a thrombin-derived oligopeptide (8), requiring a recombinant biotechnology of synthesis. Several tree species of Croton (Euphorbiaceae) are used by Indian and Mestizo populations of Peru and Ecuador for several medicinal purposes, including acceleration of wound healing. In recognition of the multiple pharmaceutical agents discovered from the Amazon rainforest, we isolated and purified taspine, an alkaloid (mol wt 369,000) extracted from the latex of *Croton lechleri*, to characterize its wound-healing properties and to define its *in vitro* and *in vivo* activity. The abundant availability of taspine and its ease of purification make it a potentially attractive alternative to growth factors.

In this study, we document a beneficial effect from a single topical application of taspine on wound healing in the paired rat surgical incision model, determine the optimal dose, and elucidate its mechanism of action by using cell culture systems. This is the first plant alkaloid confirmed to accelerate wound healing and is another example of the benefits of ethnobotany in identifying promising new pharmacologic agents.

Materials and Methods

Isolation and Purification of Taspine. Taspine extraction follows that of Bettolo and Scarpati (9), except that triethylamine (Et_3N) is substituted for diethylamine. In brief, using silica gel column chromatography, 10 ml of latex were adsorbed on 10 g of silica gel, and mixed with 10 ml of chloroform-methanol-triethylamine (9:9:2) and 18 g of silica gel. The mixture, dried *in vacuo* at 40–50°C, was eluted in a column with

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chloroform-methanol (9:1) and identified as taspine by thin layer chromatography, nuclear magnetic resonance, and mass spectrophotometry. Purity above 98% was obtained using high-performance liquid chromatography with a concentration <0.1% taspine, the compound being stable but highly insoluble, except in dimethylsulfoxide. Extracted taspine undiluted to 10^{-8} showed no detectable endotoxins, utilizing the Limulus assay.

Animals. We utilized male Sprague-Dawley rats obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN) weighing 280–320 g. The animals were maintained in in-house animal facilities with food and water *ad libitum*.

Reagents. Dimethylsulfoxide (DMSO), diaminobenzidine tetrahydrochloride, ferricytochrome *c*, and polyclonal anti-fibronectin antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Mouse anti-rat monoclonal macrophage antibody, Clone ED2 (10, 11), was purchased from Sera Laboratories (Sussex, England). An avidin-biotin kit for immunoperoxidase labeling (ABC kit) was purchased from Vector Laboratories (Burlingame, CA). [*methyl*-^{3}H]Thymidine was purchased from New England Nuclear Corp. (Boston, MA). Ficoll-Hypaque was obtained from Pharmacia Fine Chemicals (Piscataway, NJ).

In Vivo Studies

Linear Incision Model. Full thickness paired incisions of 6 cm in length were performed as described previously (2). The incisions were coapted with three surgical clips. The experimental side received taspine dissolved in 0.1 ml of DMSO and the contralateral side received 0.1 ml of DMSO as a control. Incisions were harvested at Days 5, 7, and 12 after wounding. Wound tensile strength was measured using three 8-mm strips from each wound with a tensometer (tensometer 10; Monsanto Co., St. Louis, MO).

Experimental Groups. Six experimental groups of rats were studied as described in Table I. Rats from Group 1 ($n = 10$) were used to assess the effect of the vehicle in the rat linear incision model, receiving pure DMSO (0.1 ml) in the experimental side, and an incision was performed in the contralateral side serving as control. DMSO tested separately had no effect on healing and was used as a vehicle and control. Rats from Groups 2, 3, and 4 ($n = 10$ in each group) received 250 μg of taspine dissolved in 0.1 ml of DMSO in the experimental side and vehicle (DMSO) in the control side. These groups of rats were sacrificed and skin samples were harvested at Days 5, 7, and 12, respectively. In Groups 5 and 6 ($n = 6$ in each group), taspine was tested in different concentrations (10 μg and 50 μg). These groups were harvested at Day 7 after wounding.

Table I. Experimental Groups of Rats

Group	*n*	Dose of taspine	Day of harvesting
I	10	Vehicle (DMSO 0.1 ml)	7
II	10	250 μg	5
III	10	250 μg	7
IV	10	250 μg	12
V	6	10 μg	7
VI	6	50 μg	7

Histologic and Immunohistochemical Analysis. To assess the quality and time course of the cellular inflammatory infiltration in the process of wound healing after a single topical dose of taspine, paired samples from treated and control sides were obtained for histologic examination (2). Additional samples from each side were harvested for immunoperoxidase labeling as described (12, 13). Frozen sections (8 μm) were fixed in cold acetone (4°C) for 8 min and air dried. Endogenous peroxidase activity was suppressed by immersion for 20 min in 1% H_2O_2 in phosphate-buffered saline (PBS). The sections were incubated with goat serum blocking solution (ABC kit) followed by incubation with anti-rat macrophage primary antibody diluted to 1/1000 in PBS for 1 hr. After rinsing with PBS, biotinylated anti-mouse IgG (ABC kit) was added for 30 min. After washing with PBS, slides were incubated with streptavidin peroxidase (ABC kit) and then exposed to diaminobenzidine tetrahydrochloride for 2 min. The sections were counterstained with hematoxylin and mounted under coverslips.

In Vitro Studies

These studies were performed to evaluate the influence of taspine in cell culture systems and to elucidate the mechanism of action by means of which taspine accelerates wound healing.

Fibroblast Proliferation Assay. IMR-90 cells (fetal lung fibroblasts) were plated on 30-mm plastic culture dishes (9×10^4) and allowed to adhere and spread for 12 hr. Afterward, taspine dissolved in DMSO was added to the cells at 25 or 100 μg/ml for 48 hr. Two experiments were performed in parallel. After the treatment period, the cells in one group were counted, whereas the cells in the second group were submitted to a thymidine incorporation assay.

Thymidine Incorporation Assay. This assay was performed as described (14). Briefly, IMR-90 cells were cultured for 48 hr in the presence of taspine dissolved in DMSO (25 or 100 μg/ml). [*methyl*-^{3}H]Thymidine was added for 2 hr (10 μCi/ml) and the cells were rinsed with triethanolamine-buffered saline. The labeled cells were scraped from the plate with a rubber policeman and extracted in triethanolamine-buffered saline containing 1% Triton. The material was centrifuged and the supernatant was recovered and loaded on a glass

filter, and the total amount of radioactivity was counted using a gamma counter. The glass filter was then washed with 0.1 *N* HCl to eliminate non-DNA-incorporated thymidine and filters were counted again.

Assay of Chemotaxis for Fibroblasts. Assay of fibroblast chemotaxis was performed in modified Boyden chambers, as described previously (15, 16). Fetal bovine ligament fibroblasts were obtained from explants of ligamentum nuchae as described (17). Taspine was serially diluted in DMSO and then in Dulbecco's modified essential medium before use, and five high-power fields (fibroblasts) were counted from triplicate samples. The results are reported as net cell migration.

Immunofluorescence Fibronectin Staining. IMR-90 cells were cultured in eight-chamber Lab Tek microslides (18) in the presence of 25 or 100 μg/ml of taspine for 24 hr. Afterward, the cells were rinsed, fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton, and immunostained with a polyclonal anti-fibronectin antibody (AB 1.5) (19), as described (18).

Assay of Chemotaxis for Macrophages. Chemotaxis was assessed by a modification of the method of Falk *et al.* (20) using a 48-well microchemotaxis chamber. Rat peritoneal macrophages were used as responder cells. Peritoneal macrophages from normal rats were obtained by lavage after elicitation with 0.5% shellfish glycogen and suspended in Hanks' balanced salt solution. The assay was performed as described (21). Various dilutions of taspine in DMSO in 25-μl aliquots were placed in the bottom well of the chamber. DMSO was used alone to assess random migration. To the wells in the upper chamber, 25×10^4 macrophages in a volume of 50 μl of Hanks' balanced salt solution were added. The chamber was then incubated for 3.5 hr at 37°C in a humidified atmosphere. Afterward, the polyvinylpyrrolidone-free polycarbonate filter (5-μm pore size; Nucleopore, Pleasanton, CA) separating the upper and lower halves of the chamber was removed, fixed in methanol, stained with Diff Quick, and tested in different dilutions. Results are expressed as net cells migrating through the filter per well (total cells minus random migration). Assays were performed in triplicate.

Neutrophil Assays. Human peripheral neutrophils were isolated by sequential dextran sedimentation and Ficoll-Hypaque gradient centrifugation (22). The purification procedures and protocols for handling of the cells have been outlined in detail in previously published work (23). Three different assays were performed with neutrophils.

Assay of superoxide radical generation. Superoxide production in response to cell activation by *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol myristate acetate was measured by the reduction of ferricytochrome *c* as described (23).

Assay of chemotaxis. The locomotor behavior of isolated neutrophils to 10 Nm FMLP was assessed in modified Boyden chambers (Bio-Rad), which consist of two 0.1-ml compartments separated by a methylcellulose filter of 5-μm pore size. Migration, the distance transversed by the cells in the filter, was measured by the leading-front method (24). Details of the procedures have been described previously (25).

Assay of phagocytosis. Briefly, sheep red blood cells (SRBC) were opsonized with a polyclonal rabbit antiserum in the absence of complement (26). After washing to remove excess antibody, the IgG-coated SRBC were sedimented on top of a monolayer of adherent neutrophils on a multicompartment glass slide. After 30 min at 37°C, extracellular SRBC were removed by lysis in 0.85% NH_4Cl. Thereafter, the neutrophils and engulfed erythrocytes were fixed, stained, and examined finally by light microscopy. Results are expressed as the phagocytic index, the number of ingested SRBC per 100 neutrophils. The percentage of inhibition in the presence of taspine (relative to that in the absence of drug) was calculated in the usual manner.

Calculations and Statistics

Wound Tensile Strength. For each rat, the results obtained for the wound tensile strength measurements of three samples were averaged in the experimental and control sides, respectively. Results are expressed as mean ± SE, and comparison between both sides was made by means of paired Student's *t* test. A P-value < 0.05 was considered significant.

Histologic Analysis. The cellularity of the taspine-treated and control wounds was estimated by two independent observers using a subjective scale from 1 to 4, with 1 representing baseline cellularity in unwounded dermis and 4 representing maximum cellularity at the time point tested (4, 27). The evaluation was done in a blinded manner by two independent observers, and then their results were averaged for statistical analysis (Student's paired *t* test).

Immunoperoxidase. Paired wound sections were examined microscopically, and positive staining was evaluated for the gross estimation of the number of macrophages in the granulation tissue. Calculations and statistics for *in vitro* studies are described above for each assay.

Results

The results of a single topical application of taspine (250 μg) to one side of rat linear incisions are depicted in Figure 1. A significant increase in wound tensile strength of the taspine-treated side was observed when compared with control side by Days 5 and 7 after wounding, with a 26% ($P < 0.005$) and 30% ($P < 0.0001$) increase, respectively. In contrast, no significant difference in wound tensile strength was obtained

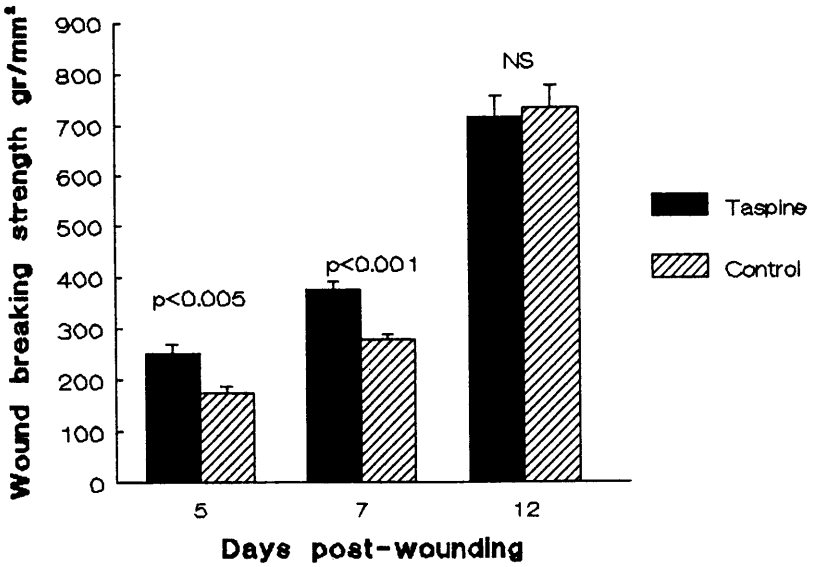

Figure 1. Wound tensile strength of linear incisions treated with 250 μg of taspine at Days 5, 7, and 12 after wounding. Taspine was topically applied at the time of wounding. For each rat, three samples were obtained from each side and tested on the tensometer for wound tensile strength. Results are expressed as mean ± SE. Values were considered significant when $P < 0.05$. NS, not significantly different.

between the experimental side receiving 250 μg of taspine and the control side in samples obtained from Day 12 after wounding. The effects of taspine in wound healing cannot be attributed to the vehicle since DMSO was devoid of any significant action when compared with nontreated paired samples; samples treated with pure DMSO had a mean tensile strength of 333.4 ± 16.3 g/m^2 in comparison with the nontreated samples, which had a mean tensile strength of 321.2 ± 29.4 g/m^2 ($n = 8$).

Taspine was also tested at doses of 10 μg/ml and 50 μg/ml applied topically at the time of wounding. No significant difference was observed between the taspine-treated and control sides (Fig. 2), indicating a lack of effect at these lower doses.

Histologic examination of samples showed a striking increase in the cellular inflammatory infiltration in wounds treated with 250 μg of taspine compared with matched control wounds by Days 5 and 7 (Table II). A higher influx of mononuclear cells was observed in samples treated with taspine in comparison with the control sides (Fig. 3). Data were analyzed and taspine-treated wounds were quantitatively compared with matched, paired control wounds (Table II). Increased cellularity influx occurred within 5–7 days after wounding in taspine-treated wounds (250 μg).

Further identification of mononuclear cells using immunohistochemical techniques with a monoclonal macrophage antibody showed no difference in the number of macrophages between the samples treated with taspine and the controls (data not shown), which suggests that the increase in inflammatory cellular infiltration produced by the topical application of taspine was not due to macrophages but rather to increased fibroblasts.

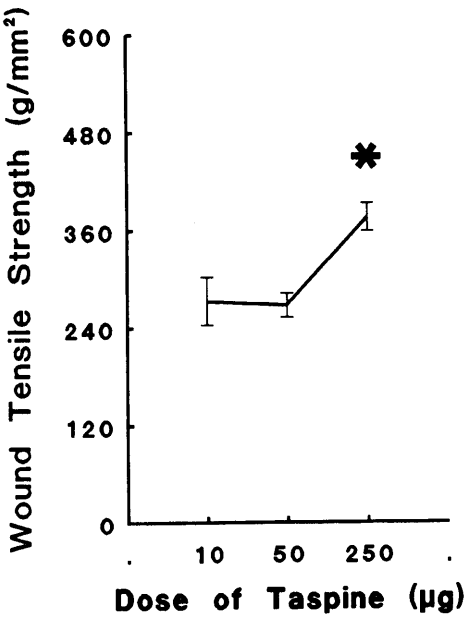

Figure 2. Dose response of wound tensile strength after application of different doses of taspine. The different concentrations of taspine were topically applied at the time of wounding. For each rat, three samples were obtained from each side at Day 7 after wounding and tested on the tensometer for the wound tensile strength. Results are expressed as mean ± SE. Values were considered significant when $P < 0.05$.

The functional activities of taspine were determined by means of multiple *in vitro* assays. The effect of taspine on fibroblast proliferation was evaluated by cell proliferation and thymidine incorporation assays.

Table II. Wound Cellularity of Taspine-Treated and Control Wounds[a]

Days after wounding	Taspine (250 μg)	Control	*t* Test
5 (n = 10)	3.30 ± 0.15	2.50 ± 0.12	$P < 0.001$
7 (n = 10)	3.10 ± 0.20	2.35 ± 0.15	$P < 0.048$
12 (n = 10)	2.15 ± 0.15	2.10 ± 0.16	NS

[a] Cellularity of paired hematoxylin and eosin histologic sections were scored by two blinded observers. A scale of 0 to 4 was used, with 0 representing baseline cellularity in unwounded dermis and 4 representing maximum cellularity at the time point tested. The blinded observations were averaged and analyzed by means of paired Student's *t* test. NS, non-significant difference in cellularity.

The results for these assays are depicted in Table III. There was no evidence suggesting a stimulatory role of taspine in fibroblast proliferation. In contrast, a decrease in cell viability and inhibition of thymidine incorporation was probably due to *in vitro* cell toxicity. These results are similar to those reported by Vaisberg *et al.* (28).

Evidence obtained *in vitro* for the increased expression of fibronectin in injured tissues and its ability to promote cell adhesion, migration, proliferation, and cytodifferentiation suggest a role for fibronectin in tissue injury and repair (29). Therefore, we examined the effects of taspine on the deposition of fibronectin by cultured lung fibroblasts. Taspine did not appear to inhibit the adhesion or spreading of IMR-90 cells to glass dishes (not shown). After 24 hr in culture in the presence of taspine, the cells were fixed and stained with anti-fibronectin antibody. Again, taspine had no discernible effects on fibronectin matrix deposition, as the treated cells showed an extensive fibronectin matrix indistinguishable from the control cells (not shown).

Chemotactic activity for fibroblasts was assayed in modified Boyden chambers. Taspine was observed to promote fibroblast migration, with the optimal cell migration at 50 pg/ml (Fig. 4). Taspine is established as chemotactic for fibroblasts in this assay.

Chemotactic activity for macrophages was assayed. Taspine did not exhibit any chemotactic properties toward macrophages; neither concentration of taspine tested showed macrophage chemotactic activity (data not shown).

The effect of taspine on several stimulated neutrophil functional responses was investigated (Table IV). Over the concentration range 3–30 μg/ml, there was a dose-dependent inhibition of the phagocytosis of opsonized sheep erythrocytes, an event mediated through the binding of immune complexes on the red cell membrane to surface Fc receptors on the neutrophil. At the highest dose tested (30 μg/ml), phagocytosis was suppressed by 70%. In sharp contrast, taspine had no appreciable effect on random migration or chemotaxis to FMLP or on the production of superoxide radicals by FMLP- or phorbol myristate acetate-activated neutrophils.

Discussion

The results of this study demonstrate that a single topical application of the alkaloid taspine at the time of wounding stimulated the critical early phases of wound tissue repair in a surgical incision model. The stimulatory effect of taspine on wound healing was dose and time dependent. A single application of 250 μg/ml/incision increased wound tensile strength by 26%

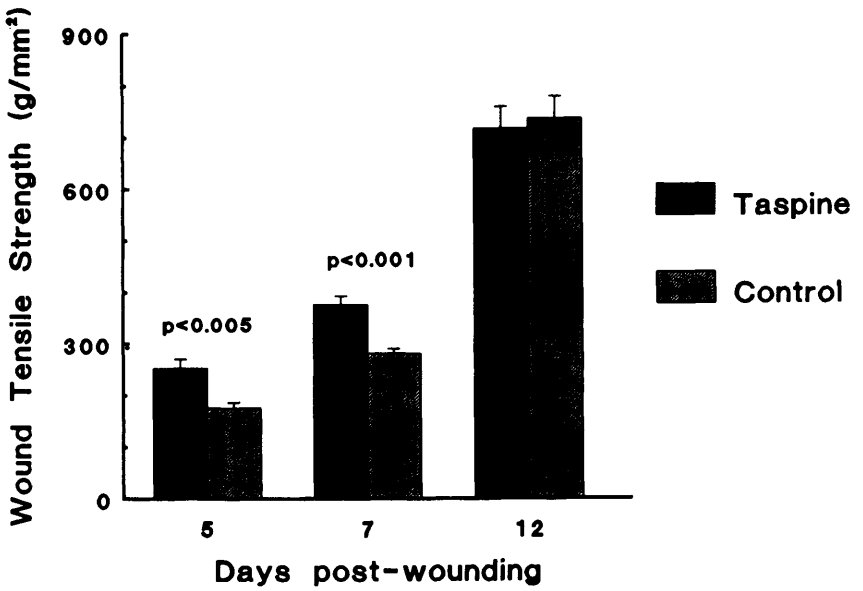

Figure 3. Histologic evaluation of paired wounds treated with taspine or vehicle. Hematoxylin and eosin staining ×150, selected samples. ■, Taspine (250 μg) at Day 7 after wounding; ▨, control wound at Day 7 after wounding. Arrows indicate limits of the granulation tissue.

Table III. Effect of Taspine in Fibroblast Proliferation

Cell proliferation assay	
Initial cell count	9×10^4
After treatment	
Control	10×10^4
Taspine 25 μg	5×10^4
Taspine 100 μg	6×10^4
Thymidine incorporation	
Total counts	
Control	2510.56
Taspine 25 μg	2482.53
Taspine 100 μg	3023.11
Incorporated counts	
Control	1360.44
Taspine 25 μg	446.99
Taspine 100 μg	372.48

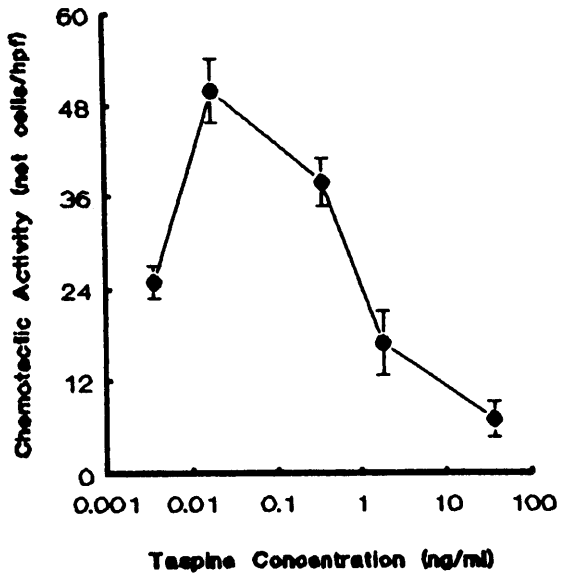

Figure 4. Chemotaxis of fibroblasts in response to taspine. Taspine was serially diluted and tested for chemotactic activity in modified Boyden chambers. The positive control of 30 ng/ml human platelet purified platelet-derived growth factor was 94 ± 4.9 cell/high-power field (hpf). Results are expressed as net cells/hpf.

and 30% at Days 5 and 7 after wounding, respectively, the time period when most surgical incision complications occur. In contrast, taspine applied at doses of 50 μg/ml/incision or 10 μg/ml/incision did not have a significant stimulatory effect on wound tensile strength. An extensive purification process was carried out to obtain taspine, a 98% pure compound, endotoxin-free, ruling out contamination or a foreign particle as responsible for the stimulatory effect of taspine in wound repair.

Virtually all complications of wound dehiscence occur within the first 7 days after surgery. During this early phase, an increase in the gain of wound tensile strength might lead to a reduction in wound complications (1, 5, 7). Although taspine did not show stimulatory effect at later time points than Day 7, it was only applied once at the time of wounding. A slow release vehicle (1) or repeated applications may be necessary for optimal activity.

Histologic analysis of skin samples from rats treated with 250 μg of taspine at the time of wounding showed a significant increase in the mononuclear cell infiltration at Days 5 and 7 after wounding when compared with controls. Qualitative analysis of the cells present in the wound using immunohistochemistry studies showed that the increased infiltration in taspine-treated wounds by mononuclear cells could not be attributed to macrophages, and so must be attributed to increased fibroblasts.

In vitro studies were carried out to evaluate the effects of taspine on the cellular elements involved in the wound-healing cascade. Assay of macrophage chemotaxis demonstrated that taspine does not possess chemotactic properties toward macrophages. This assay was consistent with the immunohistochemistry studies demonstrating no significant difference in the macrophage infiltration in samples treated with the alkaloid. These results were somewhat unexpected because it is well known that macrophages play a central role in wound healing confirmed by impaired macrophage-deficient models of healing with irradiation or steroids (5, 30, 31) and agents that enhance wound healing by increasing macrophage infiltration to the wound, such as local administration of platelet-derived growth factor (1, 3, 4, 7) and platelet-activating factor (32). However, the enhancement of wound healing that is not accompanied by a parallel macrophage infiltration is not unique to taspine. We have demonstrated that transforming growth factor-β accelerates wound repair by stimulation of fibroblasts and collagen production without increased macrophage infiltration in the incisional model (27, 30). The increased mononuclear infiltrate (fibroblasts) seen histologically can be explained by the chemotactic properties of taspine for fibroblasts *in vitro* al picomolar concentrations. No other properties, including activation of neutrophils, chemotaxis for macrophages or neutrophils, or increased extracellular matrix synthesis, were found to explain the vulnerary effects of taspine.

The results suggest that taspine is a purely chemotactic factor for fibroblasts without inflammatory or growth factor properties. Previous studies have suggested that taspine is an anti-inflammatory agent (33) and this study does not contradict our observations. The lack of a stimulatory effect in wound healing after 12 days was probably due to the absence of proliferative effects of taspine, with a temporary response to single topical application. We have demonstrated previously that purely chemotactic factors can expedite wound tissue repair by augmenting the inflammatory response (5). The present data suggest that taspine enhances wound healing, predominantly stimulating the chemo-

Table IV. Effect of Taspine on Human Neutrophil Functional Responses

Function	Stimulus	Taspine (μg/ml)	Percentage of inhibition
Phagocytosis[a]	IgG-coated SRBC	30	70
		10	41
		3	26
Chemotaxis[b]	FMLP	30	0
Superoxide generation[c]	FMLP	30	0
	Phorbol myristate acetate	30	0

[a] The phagocytic index of neutrophils not exposed to taspine was 88 ± 7. Results are means ± SE of four experiments.
[b] Without taspine, directional migration to 10 Nm FMLP by 30 min was 85 ± 6 μm into the filter. Random motility (in the absence of the stimulus) was 34 ± 3 μm. Results have been taken from three experiments.
[c] In the absence of taspine, FMLP (200 Nm)- and phorbol myristate acetate (100 Nm)-induced superoxide radical release was 10.3 ± 1.5 and 23.6 ± 4.1 nmol of cytochrome *c* reduced/10^6 neutrophils, respectively. Results shown are of three experiments at 30 μg/ml; taspine spontaneously reduced the cytochrome *c* indicator to a modest degree, thereby causing slight interference with the assay. This activity appeared to be a result of a direct chemical interaction having nothing to do with the generation of superoxide radicals, since it occurred in the complete absence of neutrophils. This background was always subtracted from the total measurements in deriving the amount of superoxide produced by the cells.

taxis of fibroblasts that migrate into the wound from local tissues and increasing extracellular matrix synthesis due to their increased number.

Although there has been a great interest in growth factors as agents to promote wound healing (1–8), none of the other described growth factors had an activity profile like taspine. Transforming growth factor-β does increase collagen deposition, and many growth factors stimulate fibroblast proliferation but have other activities as well. It is possible that taspine would act synergistically with a growth factor acting by another mechanism. The magnitude of the effect is equivalent to that seen with some growth factors (3, 8) and its potential greater availability may offer significant advantages in terms of medical utility. Further work on preparing taspine as a water-soluble salt may offer further benefits in terms of ease of administration and, perhaps, activities.

Taspine, an alkaloid extracted from *Croton lechleri*, accelerates soft tissue repair in a dose-dependent manner at initial phases of the process, probably by means of its chemotactic properties toward fibroblasts. This novel alkaloid opens a pathway of research for new tools to stimulate wound repair in the absence of macrophages, thereby helping to better understand the process of wound healing.

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