Arnebin-1 Accelerates Normal and Hydrocortisone-Induced Impaired Wound Healing[1]

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Wound healing involves inflammation, cell proliferation, matrix deposition, and tissue remodeling. Interaction of different cells, extracellular matrix proteins, and their receptors are mediated by cytokines and growth factors during wound healing. In this study, we have evaluated the effect of arnebin-1, a natural product isolated from *Arnebia nobilis,* on normal and impaired wound healing in cutaneous punch wound model. Arnebin-1 was applied topically daily on wounds of hydrocortisone-treated or untreated animals. Arnebin-1 significantly accelerated healing of wounds with or without hydrocortisone treatment as revealed by a reduction in the wound width and gap length compared with controls. Arnebin-1 treatment promoted the cell proliferation, migration, and vessel formation to form a thick granulation tissue and re-epithelialization of the wounds. An increase in the synthesis of collagen, fibronectin and transforming growth factor-β1 was seen in arnebin-1-treated wounds compared with the untreated control. As transforming growth factor-β1 is known to enhance wound healing, and associated with the wound healing defect in hydrocortisone-treated wounds, the enhanced expression of transforming growth factor-β1 at both translational and transcriptional level by arnebin-1 may be responsible for the enhancement of wound healing during normal and impaired wound repair. These studies suggest that arnebin-1 could be developed as a potent therapeutic agent for wound healing in steroid-impaired wounds. ***Key words: extracellular matrix proteins/impaired wound repair/punch wound model/transforming growth factor-β1. J Invest Dermatol 113:773–781, 1999***

Wound healing is a complicated biologic process which involves interactions of multiple cell types various cytokines, growth factors, their mediators, and the extracellular matrix proteins (ECM). Fibronectin (FN), laminin (LMN), tenascin, and collagen are extracellular matrix proteins not only involved in the early phase of the wound healing but also play an important part in structural remodeling of the tissue. ECM interaction supports the adhesion of fibroblasts and endothelial cells (Fujikawa *et al*, 1981; Hunt, 1988; Mackie *et al*, 1988; McDonald, 1989; Martin *et al*, 1992). Several cytokines/growth factors such as transforming growth factor (TGF)-β and -α, platelet-derived growth factor, epidermal growth factor (EGF), basic fibroblast growth factor, and insulin-like growth factor-1 (IGF-1) play a central part in wound healing by regulating recruitment, activation, mitogenesis, migration, angiogenesis, and differentiation of various cell types in the wound bed (Mustoe *et al*, 1987; Mooney *et al*, 1989; Falang, 1993; Brown *et al*, 1994). The use of recombinant growth factors alone or in combination has been shown to enhance the tissue repair and regeneration in animals and humans (Rothe and Flanga, 1989; Mustoe *et al*, 1991). These growth factors are mainly released from the various cells in the wound bed especially macrophages, and trigger other cells to migrate, proliferate, or produce other growth factors required for repair (Rappolee *et al*, 1988; Mckenzie and Sauder, 1990). Granulocyte macrophage-colony stimulating factor and vascular endothelial growth factor enhance wound healing in ischemic tissue by inducing vasculogenesis and neovascularization (Kalka *et al*, 1999). Thus induction of growth factors in the wound by any agent would provide better perspectives for the consideration as a potent wound healing agent.

Arnebia nobilis is one of the four species of the genus (Boraginaceae) occurring in northern India. The extract of *A. nobilis* was widely used for centuries in indigenous medicine for wound healing treatment. Investigations on the extract from the root of this plant led to isolation of four naphthaquinones designated as arnebin-1, arnebin-2, arnebin-3, and arnebin-4, which have been characterized by the Central Drug Research Institute (CDRI), Lucknow (India). Arnebin-1 was most active against Walker carcinoma in rats and P388 lymphoid leukemia in mice (Shukla *et al*, 1969) in contrary to arnebin-2 which do not have any anti-tumor activity. Arnebin-1 (5,8-dihydroxy-2-(1′-b,b-dimethylaryoxy-4′-methylpent-3-enyl)-1, 4-naphthoquinone exhibited a wide range of biologic activities such as anti-cancer, anti-bacterial, anti-fungal, anti-passive, cutaneous anaphylactic, and wound healing activities (Shukla *et al*, 1971; Katti *et al*, 1979; Painuly *et al*, 1984).

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Abbreviations: HDMEC, human dermal microvascular endothelial cells; HDF, human dermal fibroblasts; HM, human melanocytes; ICE, interleukin-1β converting enzyme; PBMC, peripheral blood mononuclear cells.

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This study was undertaken to evaluate the *in vivo* effect of arnebin-1 on wound healing. We observed a faster closure of unimpaired and hydrocortisone-impaired wounds in a full thickness punch wound model in rats as revealed by a decrease in the wound gap and wound width in arnebin-1 treated rats compared with controls. Arnebin-1 promoted the wound healing by inducing the proliferation of cells, angiogenesis, and collagen synthesis. FN and TGF-β1 were also transcriptionally upregulated in wounds by arnebin-1. The increased biosynthesis of FN, collagen and TGF-β1 by arnebin-1 may be responsible for the enhancement of wound healing.

MATERIALS AND METHODS

Animals Male Sprague–Dawley strain rats 250–300 g were purchased from Charles River laboratory. The animals were used in compliance with the US Public Health Service policy on humane care and use of animals. The animals were housed in a room with 12 h light/12 h dark cycle and were fed Purina FOX chow and water ad libitum.

Treatment Rats were randomly selected and divided into four groups: vehicle control, hydrocortisone, arnebin-1, and hydrocortisone plus arnebin-1. Arnebin-1 [0.1% aqueous suspension in phosphate-buffered saline (PBS)] was applied on the day of wounding and onwards daily for 11 d whereas the vehicle control animals received PBS on the wounds. Hydrocortisone (30 mg per kg i.m.) was given daily throughout the 11 d period. Each experiment including all the groups was done three times.

Creation of wound The animals were anesthetized using pentothal (35 mg per kg, i.p.) and hair on the dorsal side of subjects were shaved and the skin was cleaned with 70% ethanol. An 8 mm skin biopsy punch (Acuderm, Ft Lauderdale, FL) was used to create full thickness dorsal cutaneous wound under aseptic condition as described previously (Sidhu *et al*, 1998; Bharatiya *et al*, 1992). Six wounds with three on each side in each rat were created, and thereafter, the animals were individually caged.

Histopathologic studies Six rats from each group were killed on the fourth, seventh, and eleventh days after wounding using pentothal (35 mg/kg, i.p.) anesthesia. Wound tissues were excised and cut into halves; one half was collected in formalin (10% formaldehyde in PBS) for histology, hematoxylin and eosin staining, and *in situ* hybridization; the second half was quickly washed in cold sterile diethylcarbonate (DEPC)-treated PBS for RNA preparation. Serial sections (5–6 μm thick) were stained with hematoxylin and eosin for histologic evaluation and morphometry. Masson's Trichrome method for staining collagen was performed on paraffin sections followed by photomicrography.

Morphometric measurements and analysis An ocular micrometer was calibrated with the stage micrometer. Morphometric readings were noted using the ocular micrometer and then converted to actual measurements in micrometers (μm). All morphometric measurements were done from sections through the center of the wounds so as to obtain maximum wound diameter. The measurements were performed three times, by studying the slides in different random sequence, blinded to treatment. Epidermal migration was measured from the left and right margins of the wound, and the readings were added to ascertain total migration. In addition, the thickness of the newly formed epidermis was measured at 1 mm intervals, and the mean calculated. The density of granulation tissue was evaluated by averaging the number of cells in six high power fields (60 × objective), midway in the wound bed. We also counted the number of vascular spaces in six high power fields (60 × objective), midway in the wound bed. Dermal thickness was determined at the center of each section, vertically, from the surface of granulation tissue to the margin of dermis and subcutis. Eschar, where present, was not included in this measurement. Readings obtained in treated and control groups were compared and statistical analysis carried out using paired Student's t test.

Immunohistochemical staining for TGF-β and α-actin Immunostaining was performed using rabbit polyclonal TGF-β1 antibody (Santa Cruz Biotechnology, CA) by an indirect avidin–biotin–immunoperoxidase technique (Vectastain ABC Elite, Vector laboratories, Burlingame, CA) as specified by the manufacturer. Briefly, sections were placed on poly L-lysine-coated slides, deparaffinized, and hydrated. Endogenous peroxidase activity was blocked with hydrogen peroxide in methanol for 10 min. Non-specific staining was blocked with normal serum, and the sections were incubated with monoclonal anti-human TGF-β antibody for 10–16 h at 4°C. To ascertain that the reaction of antibody was specific, sections from each test were incubated with normal serum IgG separately. Slides were washed with PBS. Biotinylated secondary antibody IgG(H + L) was added for 1 h, followed by avidin–biotin–peroxidase complex for 30 min. Slides were then stained with diaminobenzidine, and counterstained with Harris hematoxylin, and eosin.

Immunostaining for α-actin was performed in mice to identify myofibroblasts in the granulation tissue, using anti-smooth muscle α-actin antibody, immunoglobulin G (IgG2a murine monoclonal, Boehringer Mannheim Biochemicals, Indianapolis, IN) by an indirect avidin–biotin–immunoperoxidase technique as described above.

Laser scan cytometry for TGF-β1 The Laser Scan Cytometer (LSC) measures multiple fluorescence and light scatter. Once the area of the tissue is gated, LSC automatically identifies cells, measures fluorescence and can differentiate between nuclear and cytoplasm fluorescence. In order to compare the relative density of intracellular immunostaining for TGF-β1 in the control and treated wounds, we performed laser scan cytometry by using LSC (CompuCyte, Cambridge, MA). Briefly, slides were deparaffinized in xylene, hydrated, and permeabilized with 0.025% saponin for 15 min, and then washed with PBS containing 0.5% bovine serum albumin. Sections were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-TGF-β (Santa Cruz Biotechnologies, CA) for 30 min. Followed by several washings, sections were counterstained with propidium iodide (PI, 2 μg per ml) to stain the nuclei containing 1 unit of RNAse for 30 min in the dark. Slides were mounted with 50% glycerol in PBS and the fluorescence signal was quantitated by LSC using CompuCyte protocol. The FITC counts were graphically represented to compare the FITC integral in the different groups.

Proliferating cell nuclear antigen (PCNA) staining in the wound PCNA is a 37 kDa molecular weight protein and is seen to be strongly associated in the nuclear region where DNA synthesis is occurring. The cells actively synthesizing DNA are detected by PCNA staining. Immunostaining was performed using monoclonal anti-PCNA antibody (Calbiochem, MA) by an indirect avidin–biotin–immunoperoxidase technique (Vectastain ABC Elite) as described above for TGF-β1 staining. The cells in the granulation tissue was evaluated for PCNA staining by averaging the number of cells in 10 high power fields (100 × objective), midway in the wound bed.

Polymerase chain reaction (PCR) analysis of mRNA transcripts of FN and TGF-β1 On the fourth day postwounding, the animals were killed, wounds were excised, washed in PBS containing DEPC, and total RNA was extracted immediately from tissues with the use of RNAzol B kit (Tel-Test, Friendwood, TX) as specified by the manufacturer. The RNA was further purified by lithium chloride precipitation, and analyzed by electrophoresis in a denaturing 1.5% formaldehyde gel to ascertain that the RNA was not degraded. RNA was reverse transcribed as described previously (Sidhu *et al*, 1998). The cDNA was amplified by PCR using sense and anti-sense specific primer. Briefly, to 3 μl of the reverse transcriptase mix, the following components were added; 10 μl of 10 × PCR buffer and 0.5 μl of Taq polymerase (5 μl); 6 μl of dNTP; 0.4 μl of sense primer (1 μg per μl) and 0.4 μl of anti-sense primer (1 μg per μl) and 79.7 μl of water. The samples were incubated in a DNA thermocycler (Gene Amp PCR system 9600, Perkin Elmer Cetus, Norwalk, CT) for 25 cycles. To confirm that equal amounts of RNA were taken in each PCR reaction in the experiment, primers for the "house keeping gene", glyceraldehyde phosphate dehydrogenase (GAPDH) were used for comparison. The primers used for DNA amplification were specific for TGF-β1, FN, and GAPDH to the rat sequence. The location of primers for nucleotide sequence amplification of amplified DNA product on southern blot were bases: 1271–1292 (sense), 1510–1491 (anti-sense), and 1401–1421 (probe) for TGF-β1; 1657–1677 (sense) and 2070–2050 (anti-sense), and probe (1830–1850) for FN; 388–405 (sense), 581–562 (anti-sense), and 531–549 (probe) for GAPDH. The sequences for anti-sense probes were selected from cDNA for TGF-β1, FN, and GAPDH, were examined for sequence homology in the GENE BANK, and were found to be gene specific.

The final PCR products from each sample were run on 1% GTG agarose (Seakem, Rockford, ME), blotted to nytran membrane (Schleicher and Schuell, Keene, NH) and hybridized to ^{32}P (NEN, Boston, MA), end-labeled oligoprobes at 42°C for 8 h and autoradiographed.

***In situ* hybridization** This was performed as described (Diamond *et al*, 1990) with slight modifications. Briefly, the slides were fixed in 4% paraformaldehyde and acetylated for 10 min, dehydrated, and hybridized for 18 h at 42°C. Plasmid PBR322 containing murine cDNA was a

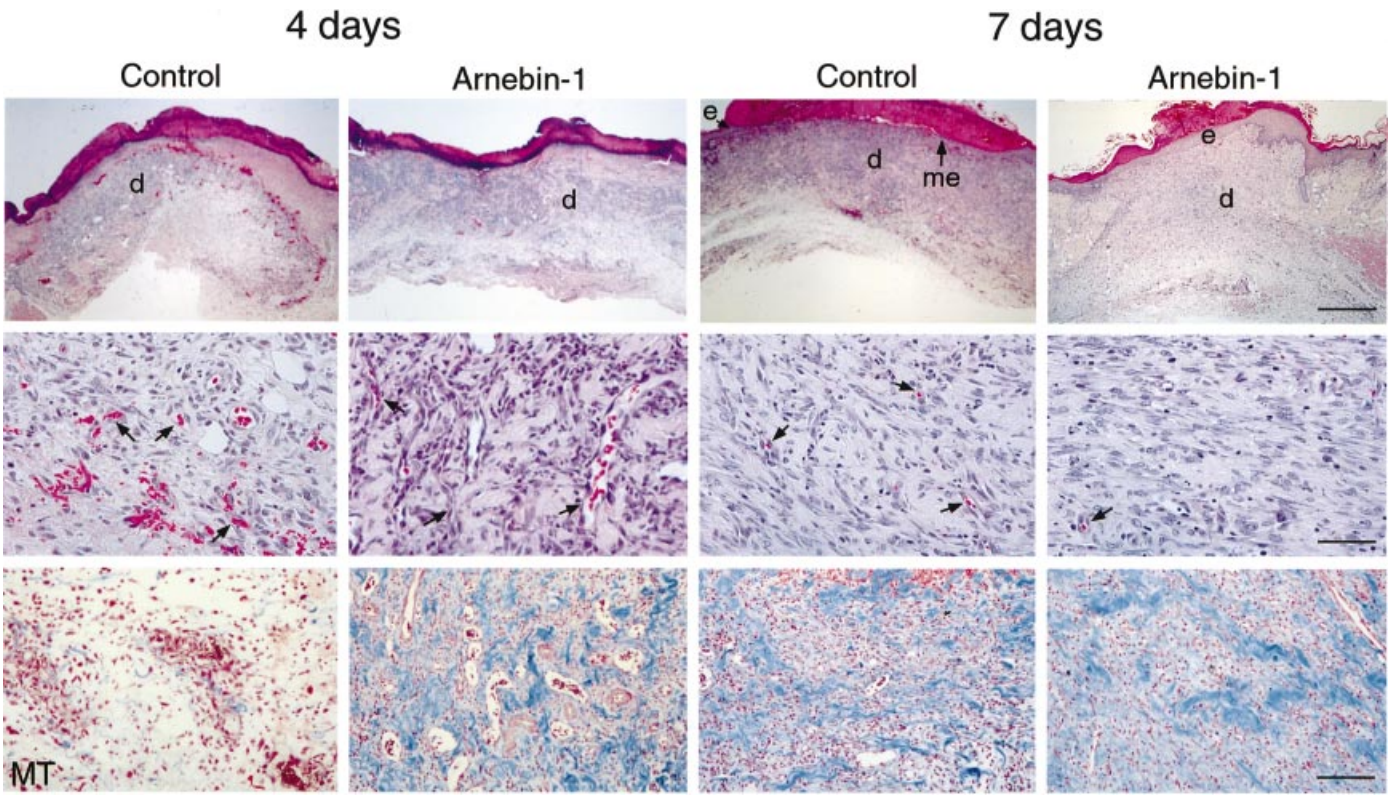

Figure 1. Arnebin-1 enhanced wound healing in rats. Vehicle control and arnebin-1-treated hematoxylin/eosin-stained sections of fourth and seventh day punch wounds. A loose crust over the epithelial loss was seen in both control and arnebin-1-treated wounds at 4 d postwounding. *Middle panel* is the magnified dermis near dermal and epidermal junction. An increase in the number of cells and blood vessels are seen in the dermis. Higher magnification of dermal area, showing an increase in the number of cells and blood vessels in arnebin-1-treated wounds compared with controls. Seven day control wounds showing migrating epithelium over the dermis. Arnebin-1 treatment resulted in complete re-epithelialization of the wounds by the seventh day. Masson's trichrome (MT) staining shows collagen in blue. Arnebin-1 treatment enhanced collagen production on both the fourth and seventh days postwounding. *Scale bar*: (*top panels*) 50 μm; (*middle panels*) 5 μm; (*bottom panels*) 10 μm. e, epidermis; d, dermis; me, migrating tongue of the epithelium. *Arrows* show the blood vessels.

generous gift from Dr Helen Coon, USUHS, Bethesda, MD. Murine cDNA insert was excised, and purified murine cDNA insert of TGF-β was labeled with [α-^{35}S] dCTP (ICN Biomedical, Irvine, CA) (1000 Ci per mmol) with random primers. Hybridization was performed using solution containing 0.1 mg ^{35}S-labeled cDNA probe per ml, 10 mM dithiothreitol, 500 mg acetylated bovine serum albumin per ml, 0.3 M sodium chloride, 50% deionized formamide, 10% (wt/vol) dextran sulfate, 500 mg per ml tRNA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 1 mM ethylenediamine tetraacetic acid, and 50 mM Tris/HCl, pH 7.6. Posthybridization was performed by sequential incubation of the slides in a progressively more dilute salinecitrate solution with 5 mM dithiothreitol and 0.1% Triton-X, dehydrated, and dipped in NTB3 emulsion for autoradiography. After exposure for 7–10 d at 4°C, the slides were developed with D19 developer, stained with eosin and mounted with permount. The cells were examined and photographed with a Zeiss microscope equipped with camera attachments.

Masson's Trichrome and immunohistochemical staining, and *in situ* hybridization were performed on eight to 10 wounds with 10–12 serial sections of each wound to assess the reproducibility of the wounds.

RESULTS

Morphologic changes in arnebin-1 treated and the control wounds Multiple cross-sections of hematoxylin and eosin stained sections of arnebin-1 treated and untreated biopsy punch tissues of rats were examined for epithelial regeneration, infiltration of cells, density of newly formed vessels, and the organization of the granulation tissue. Wound organization was assessed in terms of degree of collagenization and alignment of collagen fibers parallel to the skin surface. Arnebin-1 treatment enhanced wound healing in both unimpaired and hydrocortisone-impaired wounds (**Figs 1** and **2**). On the fourth day postwounding (**Fig 1**), there was no or minimal epidermal migration in the control wounds and the underlying dermis showed ill-formed granulation tissue with a few newly formed capillary channels, and sparse collagen. Arnebin-1-treated wounds show more number of cells and vessels in the granulation tissue. On the seventh day postwounding, vehicle-treated control wounds showed epithelial migration over the dermis whereas the arnebin-1-treated wounds showed a well-formed and differentiated epidermis. Organization of the granulation tissue was also more advanced with thick bundles of well-aligned collagen in arnebin-1-treated wounds.

The epidermal regeneration was enhanced by arnebin-1 in hydrocortisone-treated wounds on the fourth and eleventh days postwounding (**Fig 2**). On the fourth day postwounding, arnebin-1 treatment in hydrocortisone-treated rats in resulted the formation of dense granulation tissue with arborizing capillary channels, whereas inflammatory cells and a few vessels were seen in the granulation tissue of controls. Arnebin-1 treatment induced the collagen synthesis in hydrocortisone-treated wounds. Wounds on the eleventh day were much healthier in arnebin-1-treated groups. Organization of the granulation tissue was also more advanced in arnebin-1-treated wounds with thick bundles of well-aligned collagen compared with controls.

Morphometric analysis of the histologic sections of the wounds confirmed that arnebin-1 enhanced the formation and migration of the epidermis over the wound (**Table I**). Quantitation of epidermal migration in arnebin-1-treated wounds showed a statistically significant increase in migration of epidermal tongues as compared with controls. Arnebin-1 treatment resulted in the formation of thick epidermis in normal wounds compared with controls and hydrocortisone-treated wounds. There was no apparent difference in the thickness of migrating epidermis between either of hydrocortisone alone or hydrocortisone and arnebin-1-treated wounds (**Table I**), suggesting that steroids did not affect the thickness of the epidermis.

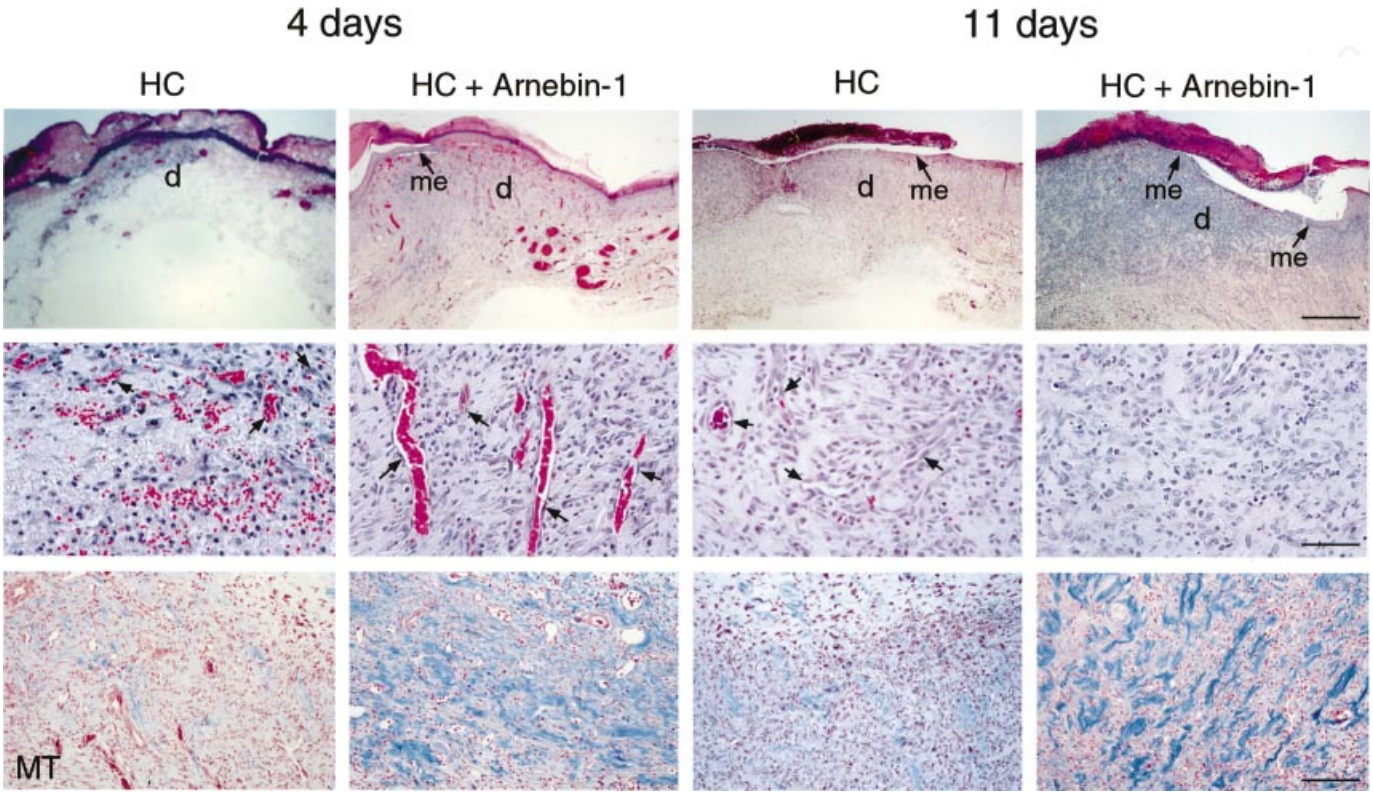

Figure 2. Arnebin-1 enhanced wound healing in hydrocortisone (HC)-treated rats. Hematoxylin/eosin-stained sections of fourth and eleventh day punch wounds. A loose crust over the epithelial loss was seen in control and arnebin-1-treated wounds at 4 d postwounding. Migrating epithelium is seen in arnebin-1-treated wounds. *Middle panel* is the magnified dermis near dermal and epidermal junction. Inflammatory cells and neovascularization is seen at fourth day control wounds in the dermis, whereas the dermal area is infiltrated with a large number of cells and vessels in arnebin-1-treated wounds. Eleven day hydrocortisone-treated wounds shows epithelial regeneration over the dermis, and immature granulation tissue infiltrated with a large number of cells and mild neovascularization. Arnebin-1 treatment in hydrocortisone-treated wounds, showing migrated epithelium, and matured granulation tissue with the disappearance of the microvessels. Masson's trichrome (MT) staining shows collagen in blue. Arnebin-1 treatment enhanced collagen production compared with control wounds Collagen was loosely arranged in the control whereas well aligned collagen fibres are seen in arnebin-1-treated wounds. *Scale bar*: (*top panels*) 50 μm; (*middle panels*) 5 μm; (*bottom panels*) 10 μm. *Arrows* shows the vessels. e, epidermis; d, dermis; me, migrating tongue of the epithelium. *Arrows* show the blood vessels.

Table I. Epithelial migration from the wound edge and thickness of the epithelial layer on the seventh day postwounding[a]

Treatment	Epithelial migration (μm)	Thickness of the epidermis (μm)
Vehicle control	2423 ± 121	120 ± 24
Arnebin-1	4508 ± 267[b,d]	288 ± 46[a]
HC	1467 ± 115[b]	138 ± 19
HC + arnebin-1	3532 ± 327[b,c]	151 ± 29

[a]Epithelial migration from both the edges of the wound was measured with ocular micrometer using microscope. Each measurement is average of 36 wounds and the values are expressed as mean of epithelial migration ± SEM or thickness of the epithelium ± SEM.
[b]$p < 0.001$; significant difference from vehicle control.
[c]$p < 0.001$; significant difference from hydrocortisone treated controls) (Student's t test).
[d]Ninety per cent of animals treated with arnebin-1 showed complete re-epithelialization of the wound. HC, hydrocortisone.

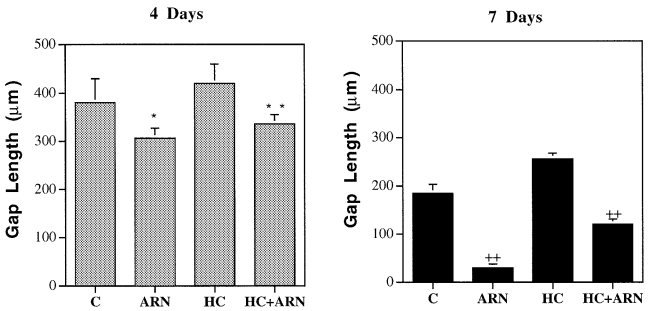

Figure 3. Arnebin-1 increased gap closure during normal and impaired healing. Arnebin-1 treatment increased gap closure (re-epithelialization) in both control and hydrocortisone-treated wounds on the fourth day postwounding. This trend continues and on the seventh day, the arnebin-1-treated wounds were almost completely closed in normal healing, whereas the wounds were not completely closed in impaired healing; however, the gap closure was significantly increased in arnebin-1-treated wounds compared with hydrocortisone-treated controls. Measurements are expressed as the mean of gap length ± SEM, n = 18. (*$p < 0.001$, significant difference from vehicle control, **$p < 0.001$, significant difference from hydrocortisone-treated control, $^{++}p < 0.0001$, significant difference from normal and hydrocortisone-treated control, Student's t test). C, control; ARN, arnebin-1; HC, hydrocortisone.

Re-epithelialization and contraction of treated wounds Wounds treated with arnebin-1 showed a statistically significant decrease in the epidermal gap between the migrating tongues of epidermis as compared with vehicle control and hydrocortisone-treated wounds (**Fig 3**). This increase in the re-epithelialization was also observed in wounds treated for 7 d and the rate of gap closure was slightly accelerated over that observed at day 4. The rate of re-epithelialization in hydrocortisone-treated wounds, however, was slower as compared with control wounds.

Contraction (width) of the wounds was also evaluated at both 4 and 7 d. After 4 d of arnebin-1 treatment, wounds showed a statistically significant difference in the decrease of wound width in both normal and hydrocortisone-treated wounds as compared with controls (**Fig 4**). The decrease in the wound width was also observed at day

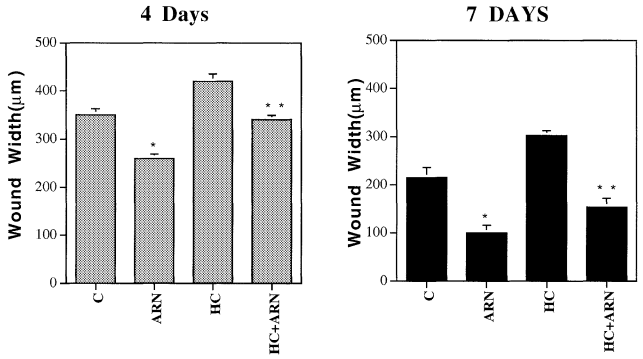

Figure 4. Arnebin-1 reduced wound width in normal and impaired wounds. Arnebin-1 treatment decreased the wound width (contraction of dermis) of normal and hydrocortisone-treated wounds on both the fourth and seventh day postwounding. Measurements are expressed as the mean of wound width ± SEM, n = 12. (*$p < 0.001$, significant difference from vehicle control, **$p < 0.001$, significant difference from the hydrocortisone-treated control, Student's t test). C, control; ARN, arnebin-1; HC, hydrocortisone.

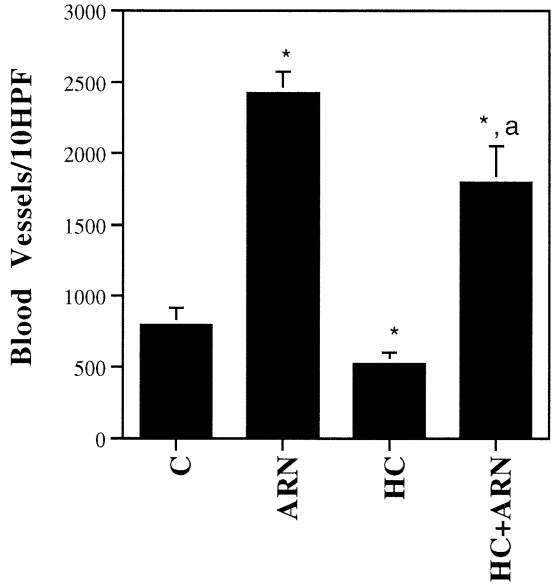

Figure 5. Density of vessels in the granulation tissue on the fourth day postwounding. Vascular spaces as identified by endothelial-lined channels were counted in the entire cross-section of the wound bed. The mean numbers of blood vessels per 10 high power fields (40×) were calculated for each wound and averaged for the group (i.e., control or treated). Vessel counts are significantly higher in arnebin-1-treated wounds on the fourth day postwounding as compared with controls showing a richer granulation tissue formation after arnebin-1 treatment. Values are expressed as mean number of vessels ± SEM. Student's t test was applied for statistical analysis. (*$p < 0.001$, n = 12 significant difference from control, and [a]$p < 0.001$, n = 12 significant difference from hydrocortisone-treated control). C, control; ARN, arnebin-1; HC, hydrocortisone.

7. These data demonstrate that arnebin-1 treatment increases wound re-epithelialization and contraction, suggesting that arnebin-1 enhanced wound closure in both normal and healing-impaired animals.

Arnebin-1 treatment enhanced the formation of granulation tissue Granulation tissue is composed of new blood vessels, collagen, and several cell types including fibroblasts, myofibroblasts, and macrophages. We investigated whether the healing wounds showed any increases in the angiogenesis by counting the blood vessels in the dermis. Vessel counts showed a significant increase (4-fold) in the number of vessels in arnebin-1-treated wounds on the fourth day postwounding in both normal and impaired healing animals (**Fig 5**),

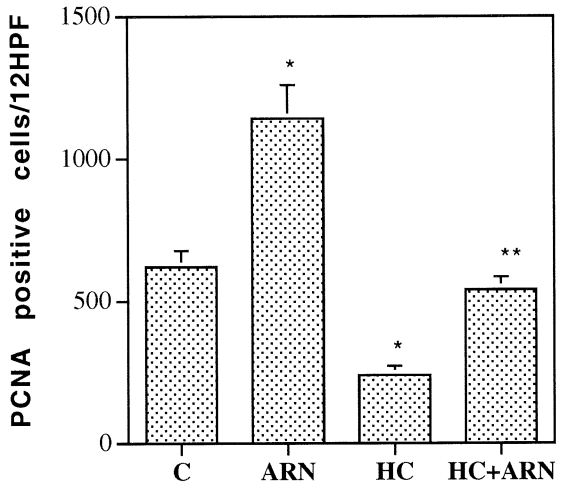

Figure 6. Arnebin-1 increased proliferating cells in the granulation tissue at the fourth day postwounding. PCNA staining was performed in paraffin-embedded sections of the wounds and the PCNA positive cells were counted in the entire section of the wound. The mean numbers cells per 12 high power fields (100×) was calculated for each wound and averaged for the group (i.e., control or treated). Arnebin-1 treatment significantly increased the number of proliferating cells in the granulation tissue of both normal and impaired wounds. Values are expressed as the mean number of PCNA positive cells ± SEM. Student's t test was applied for statistical analysis. *$p < 0.0001$, significant difference from control wounds, and **$p < 0.001$, difference from hydrocortisone-treated controls. C, control; ARN, arnebin-1; HC, hydrocortisone.

indicating arnebin-1 treatment resulted in considerable capillary ingrowth.

The migration and the proliferating of cells into the wound bed promote wound healing by secreting various growth factors and extracellular matrix proteins. PCNA staining showed an increase in the proliferating cells in the granulation tissue in arnebin-1-treated wounds compared with controls during normal and impaired healing (**Fig 6**), suggesting that arnebin-1 facilitated the infiltration of proliferating cells into the wound bed. Arnebin-1-treated wounds consistently showed a greater influx of cells. To determine whether the infiltrating cell population contains myofibroblasts, sections were stained with SM α-actin. Many of the proliferating cells in the control and arnebin-1-treated wounds were positive for SM α-actin staining, indicating the presence of myofibroblasts in the granulation tissue of the wounds (**Fig 7**). Arnebin-1-treated wounds showed an increase in the number of myofibroblasts compared with controls. The increase in the number of myofibroblasts, and the accumulation/biosynthesis of collagen in arnebin-1-treated wounds supports the observation of decreased wound width, suggesting a role for arnebin-1 in wound contraction.

Effect of arnebin-1 on TGF-β1 induction TGF-β1 was localized in arnebin-1-treated and untreated wounds. The brown color indicates the staining for TGF-β1. A large number of cells especially the fibroblasts show staining for TGF-β1 in arnebin-1-treated wounds compared with controls (**Fig 8**). On the seventh day postwounding, the epidermis of the arnebin-1-treated wounds showed staining for TGF-β1, whereas no staining was observed in the epidermis of the controls (**Fig 8**).

This was further brought out by FITC integral analysis on laser scan cytometry following immunofluorescence staining (**Fig 9**). The same site of the control and arnebin-1 treated wound was gated for the quantitation of the expression of TGF-β1 using LSC. The left panel of **Fig 8** shows the intensity of the PI indicating the number of cells in the control and arnebin-1-treated wounds. The right-hand panel (**Fig 9**) shows the amount of fluorescence obtained from FITC and represents the population of cells positive for TGF-β1. As evident from **Fig 9**, arnebin-1 treatment increased the expression of TGF-β1 along with the cell population in granulation tissue.

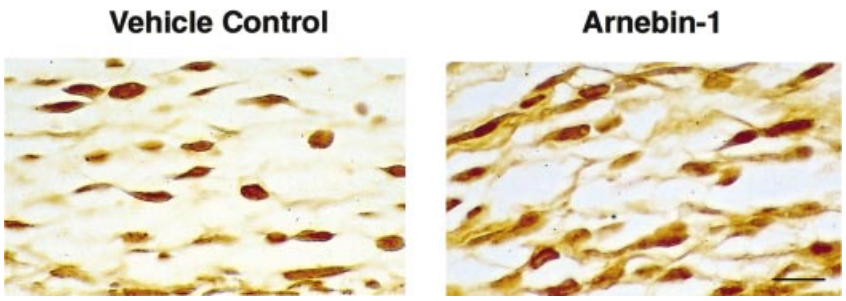

Figure 7. SM α-actin staining in fourth day rat wounds. Immunostaining was performed using anti-α-smooth muscle (SM)-actin antibody, immunoglobulin G (IgG2a murine monoclonal) by an indirect avidin–biotin–immunoperoxidase to look for myofibroblasts. Arnebin-1 treated wound, showing a larger number of positive staining cells compared with control. *Scale bar*: 2.5 μm.

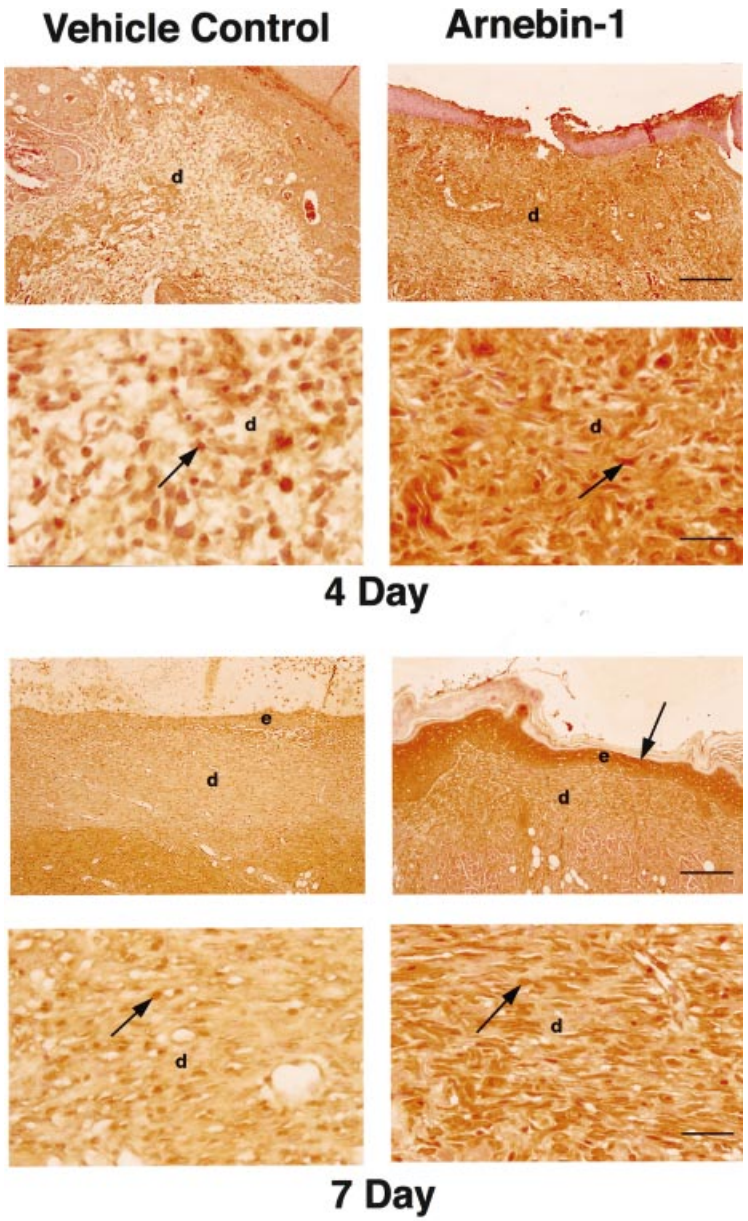

Figure 8. Immunohistochemical staining for TGF-β1 in wounds. Immunostaining was performed using rabbit polyclonal antibody to TGF-β1 (SantaCruz Biotech, CA), and avidin–biotin–immunoperoxidase, Vectastain ABC Elite kit. Fourth day (*top panel*)and seventh day (*bottom panel*) post-wounding. Arnebin-1-treated wounds show more numbers of positively stained cells as well as a greater intensity of staining at both fourth and seventh day postwounding. Arnebin-1-treated wounds show an increased staining for TGFβ1 in the epidermis compared with that of control wounds. d, dermis; e, epidermis. *Scale bar*: (*top panels*) 50 μm; (*bottom panels*) 5 μm for each 4 and 7 day wounds. *Arrows* indicate the cell showing TGFβ1 expression.

Arnebin-1 increases mRNA transcripts of FN and TGF-β1 Growth factors and ECM proteins play important parts in many phases of wound healing; therefore, we selected TGF-β1 from growth factors and FN from ECM. To determine whether arnebin-1 treatment was enhancing the biosynthesis of TGF-β1 and FN, we examined their transcripts using *in situ* hybridization and reverse transcription–PCR. An increase in the mRNA transcripts of FN (130% + 48%) and TGF-β1 (117% + 32%) was observed in arnebin-1-treated wounds compared with vehicle control (**Fig 10**), suggesting that arnebin-1 enhanced the expression of both TGF-β1 and FN in the wounds.

In an another experiment, we localized the mRNA expression of TGF-β1 by *in situ* hybridization on paraffin-embedded tissue sections from the wounded area. A few grains were observed in the vehicle-treated wounds when the tissue was hybridized with the cDNA of TGF-β1, indicating the basal level presence of TGF-β1 during normal healing (**Fig 11*a***). A large number of grains were present in arnebin-1-treated wounds indicating an increased

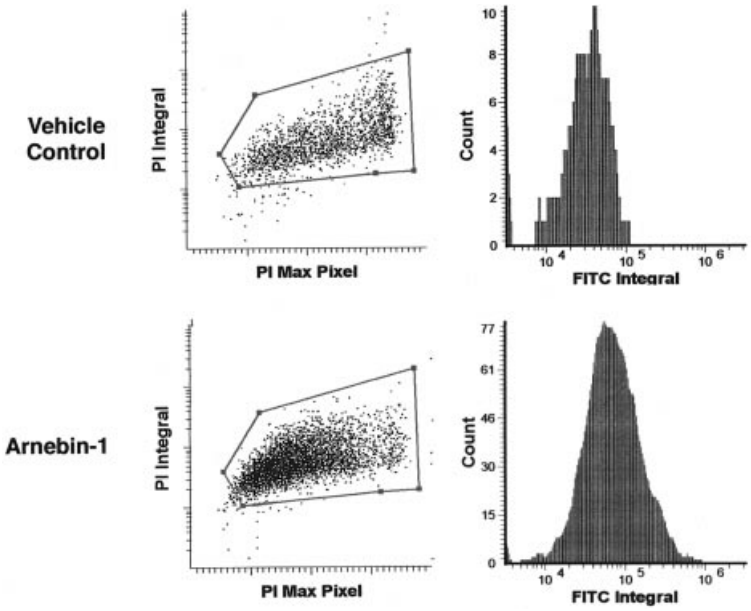

Figure 9. Quantitative analysis of TGF-β1 expression by LSC in fourth day wounds Paraffin-embedded sections were processed, incubated with FITC-conjugated-anti-TGF-β1 antibody for TGF-β1 staining in the cytoplasm of the cells, and counterstained with PI for nuclear staining. Similar sites from the granulation tissue of the control as well as arnebin-1-treated wounds were gated for measuring the fluorescence. *Left panel* shows the PI staining representing number of cells. *Right panel* shows the FITC staining for TGF-β1 *versus* number of cells from the gated area. As it is evident, the arnebin-1-treated wounds resulted in the increase of TGF-β1 positive cells compared with control; however, the TGF-β1 expression was several times more compared with the increase in the number of cells, indicating that arnebin-1 enhanced the expression in the individual cells compared with controls.

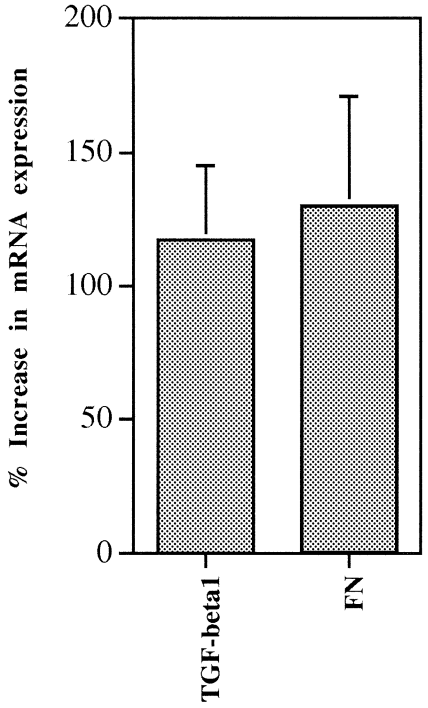

Figure 10. mRNA transcripts of FN and TGF-β1 in fourth day wounds of rats. Reverse transcription/PCR product was blotted and southern hybridization was performed using ^{32}P oligos probe for FN or TGF-β1 or GAPDH. GAPDH was used as an internal control. PCR bands were quantitated in NIH image program (Version 1.5.9) using the gel plotting macros. The signals for TGF-β1 and FN were normalized for the GAPDH and the percentage increase was calculated. The results are expressed as a percentage increase ± SEM.

expression of TGF-β1 as compared with vehicle-treated wounds (**Fig 11*a***). Higher magnification of the dermis revealed that the prominent cells showing hybridization were macrophages in both control and arnebin-1-treated wounds (**Fig 11*b***).

DISCUSSION

Wound healing is a highly ordered and well coordinated process that involves inflammation, granulation, fibrogenesis, neovascularization, wound contraction, and resurfacing of the wound defect with the epithelium. Wound healing is not a simple linear process in which growth factors trigger cell growth, but is an integration of dynamic interactive processes involving cells, ECM and their receptors, and soluble mediators (Clark, 1993). This is especially true of healing by secondary intention as occurs in a nonincisional wound spanning a large surface area. Thus the punch wound model is a desirable method to evaluate the various parameters involved in the biology of wound repair.

Glucocorticoids constitute a potent class of anti-inflammatory compounds. The adverse effects of glucocorticoid therapy is the inhibition of wound healing in humans and other animal models. The mechanisms of improvement are not entirely clear but several forms of impairment result from a decrease in the proliferation of inflammatory cells or a delay in the inflammatory phase of wound healing and consequently inadequate tissue formation. Formation of healthy granulation tissue with neovascularization is a prerequisite for healing. Also, this granulation tissue provides the medium for epidermal cells from the wound margin to migrate and bridge the gap. Arnebin-1 treatment resulted in an increased formation of granulation tissue, including greater cellular content and neovascularization, and a faster re-epithelialization of the wound in both normal and impaired punch wounds as compared with their respective control wounds, demonstrating that arnebin-1 treatment enhanced wound healing. During granulation tissue formation, as the contraction proceeds and resistance increases, fibroblasts differentiate into myofibroblasts (Skalli and Gabbiani, 1988; Rudolph *et al*, 1992; Petroll *et al*, 1993). The presence of myofibroblasts is considered to be the characteristic of tissue undergoing contraction (Darby and Gabbiani, 1990; Desmouliere *et al*, 1993). The abundance of myofibroblasts in arnebin-1-treated wounds, signifies the advanced stage of healing in these wounds as compared with controls.

The process of postinflammatory wound healing and regeneration is characterized by a complex, multicomponent cascade of degradative and biosynthetic reactions that direct underlying cell–cell and cell–ECM interactions (Hunt, 1988). ECM proteins are well known to be important in many phases of wound healing. FN helps in the phagocytosis of wound debris and promotes the migration of wound fibroblasts, endothelial cells, and epithelial cells over the wound. Collagen type III is increased in the early granulation tissue and is replaced by collagen type I (Kirsner and Eaglestein, 1993). These two collagens impart strength to the healing wound. Collagen and FN are also chemotactic for fibroblasts. Increased expression of collagen and FN by arnebin-1 suggested that arnebin-1 indirectly helped in the remodeling and regeneration of tissues as well as the provision of strength to the wounded tissues.

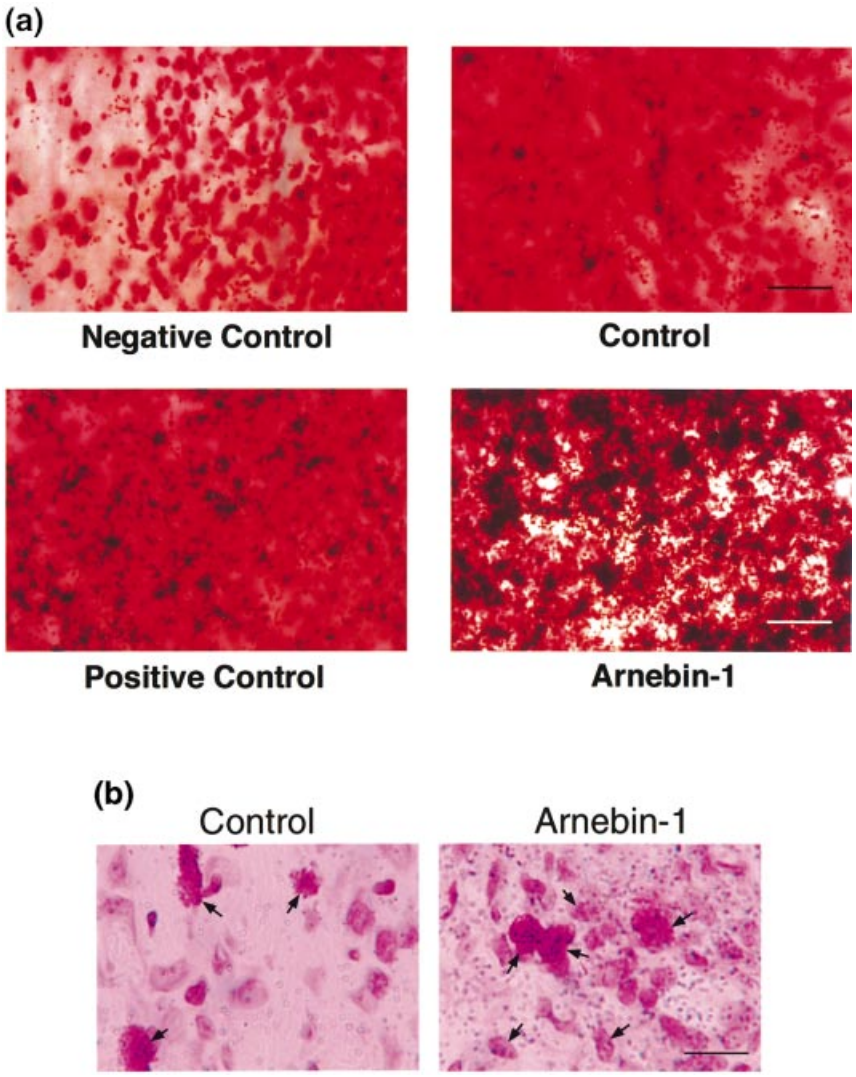

Figure 11. *In situ* hybridization for mRNA TGF-β1 in fourth day wounds. (*a*) Black grains indicate mRNA hybridization for TGF-β1. Negative control: wound section hybridized with plasmid vector pBR322 from which TGF-β1 cDNA was excised. Positive control: a wound treated with a known inducer (curcumin) of TGF-β1. Lack of grains on hybridization with a plasmid in the negative control, and a large number of grains present in the positive control indicate the degree of specificity of the probe. Low number of silver grains in the control wound, and a large number of silver grains in arnebin-1-treated wound, indicated that arnebin-1 treatment induced the mRNA expression of TGF-β1. Photomicrographs were taken of the granulation tissue near the dermal and epidermal junction. *Scale bar*: 50 μm. (*b*) Higher magnification of the dermal region shows that the signal intensity is seen mainly in macrophages, and is prominent in arnebin-1-treated wounds. *Scale bar*: 10 μm. *Arrows* indicate the cell showing mRNA expression for TGF-β1.

The peptide growth factors, cytokines and their receptors, which are expressed by various cell types at the site of injury, play a key part in regulating repair processes. This regulation is highly complex, involving the individual action of and/or synergistic interactions among many substances. These include various members of the growth factor family/cytokines, such as basic fibroblast growth factor, platelet-derived growth factor, IGF-1, TGFα, and TGF-β. Growth factors have improved healing in animals impaired with diabetes, malnutrition, infection, hypoxia, hydrocortisone, and radiation (Greenhalgh, 1996). The role of TGF-β has been suggested during impaired wound healing. The TGF-β belong to a superfamily of proteins that regulate cellular growth, differentiation, angiogenesis, chemotaxis, cell migration, and ECM (O'Kane and Ferguson, 1997). TGF-β1 is a very potent stimulant for collagen deposition and inhibits collagen breakdown by the induction of a tissue inhibitor of metalloproteinase (Laiho *et al*, 1987). TGF-β1 increases the rate of formation of granulation tissue *in vivo* and stimulates epidermal migration and epidermis regrowth (Mustoe *et al*, 1987; Pierce *et al*, 1989). Particle-mediated gene transfer with TGF-β1 cDNA enhances wound repair in rat skin (Benn *et al*, 1996). A reduced endogenous production of TGF-β1 was found in wounds of adramycin-treated animals and the supplementation of the exogenous TGF-β1 accelerates the impaired healing wounds (Lawrence *et al*, 1986).

Glucocorticoids suppressed TGF-β1, TGF-β2, and TGF-β tIIrc in normal and wounded skin (Frank *et al*, 1996). Dexamethasone abrogates the fibrogenic effect of TGF-β1 in rat granuloma and granulation tissue fibroblasts (Meisler *et al*, 1997). An increased expression of interleukins-1α and -1β, and tumor necrosis factor α has been demonstrated during normal repair and this induction was significantly lower in healing-impaired glucocorticoid-treated animals (Hubner *et al*, 1996). Recently, it has been shown that systemic glucocorticoids treatment caused a severe defect in wound repair that was accompanied by reduced expression of platelet-derived growth factor A and B and of B type receptor in the early phase of wounding (Beer *et al*, 1997). These altered balances may underlie healing defects associated with steroids use and may explain beneficial effects of TGF-β1 therapy in improving wound healing in glucocorticoid-treated animals (Slavin *et al*, 1992). TGF-β1 can prevent methylprednisolone-induced inhibition of wound healing (Osaka, 1997). *In vitro* experiments have also revealed that TGF-β1 can partly counteract the action of glucocorticoids, providing molecular interpretations for the suppression of wound healing (Fassler *et al*, 1996) and thus, TGF-β1 has critical regulatory roles in tissue repair and remodeling. Increased transcription of TGF-β1 in cells of the wound bed may be responsible for the higher levels of TGF-β1 in the wound tissue. The endogenous production of TGF-β1 by arnebin-1 could lead to the increased granulation tissue deposition and faster re-epithelialization, and may partly be responsible for improved healing in hydrocortisone-treated animals.

Arnebin-1 has been demonstrated to be active also against Walker carcinosarcoma in rats (Gupta and Mathur, 1972). Wound healing properties, however, are not normally associated with anti-cancer activity, as several anti-tumor agents such as adriamycin, actinomy-

cin D, and 5-fluorouracil do not display wound healing properties (Cohen *et al*, 1975). The wound healing activity of the naphthaquinone pigments (esters of alkannin) from plant *Alkanna tinctoria* was examined in a clinical study on 72 patients suffering from ulcus cruris (Papageorgiou, 1978). The proliferative growth of granulation tissue, as well as epithelialization of the edges of the ulcer, were observed in patients treated with naphthaquinone pigments. A treatment of 5–6 wk resulted in complete healing. In the present study, arnebin-1, a naphthaquinone displayed a remarkable ability for tissue repair and regeneration in impaired healing; therefore, arnebin-1 or its analogs may well prove to be of considerable therapeutic value.

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