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Modulation of Inflammation by Cicaderma Ointment Accelerates Skin Wound Healing

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ABSTRACT

Skin wound healing is a natural and intricate process that takes place after injury, involving different sequential phases such as hemostasis, inflammatory phase, proliferative phase, and remodeling that are associated with complex biochemical events. The interruption or failure of wound healing leads to chronic nonhealing wounds or fibrosis-associated diseases constituting a major health problem where, unfortunately, medicines are not very effective. The objective of this study was to evaluate the capacity of Cicaderma ointment (Boiron, Lyon, France) to accelerate ulcer closure without fibrosis and investigate wound healing dynamic processes. We used a necrotic ulcer model in mice induced by intradermal doxorubicin injection, and after 11 days, when the ulcer area was maximal, we applied Vaseline petroleum jelly or Cicaderma every 2 days. Topical application of Cicaderma allowed a rapid recovery of mature epidermal structure, a more compact and organized dermis and collagen bundles compared with the Vaseline group. Furthermore, the expression of numerous cytokines/molecules in the ulcer was increased 11 days after doxorubicin injection compared with healthy skin. Cicaderma rapidly reduced the level of proinflammatory cytokines, mainly tumor necrosis factor (TNF)-α and others of the TNF pathway, which can be correlated to a decrease of polymorphonuclear recruitment. It is noteworthy that the modulation of inflammation through TNF-α, macrophage inflammatory protein-1α, interleukin (IL)-12, IL-4, and macrophage–colony-stimulating factor was maintained 9 days after the first ointment application, facilitating the wound closure without affecting angiogenesis. These cytokines seem to be potential targets for therapeutic approaches in chronic wounds. Our results confirm the use of Cicaderma for accelerating skin wound healing and open new avenues for sequential treatments to improve healing.

Introduction

Skin wound healing involves a series of complex processes that need the interaction of cytokines and growth factors produced by many different specialized cells. During normal wound healing, these orderly events can be classified in four overlapping phases: inflammation, formation of granulation tissue, re-epithelialization, and matrix formation/remodeling. When these stages are delayed for more than a few weeks, wound consequently heals unusually slowly, such as in diabetic foot ulcer (Jeffcoate and Harding, 2003) or more generally necrotic ulcer (Disa et al., 1998). This defines clinically the chronic wound, one of the most common disorders, which severely impairs the quality of life of the patient and creates a huge financial burden on the healthcare system. Classically caused by a variety of events such as trauma, exposure to heat, cold, corrosive material, or radiations, and problems with blood circulation, skin ulcers are open wounds often accompanied by the sloughing of inflamed tissue. To improve this situation, many different therapeutic approaches have been tested to accelerate healing processes without fibrosis in the scar. For example, in several animal models for wound repair, a significant increase of healing was obtained by topic application of growth factors such as keratinocyte growth factor (Shannon et al., 2006; Henemyre-Harris et al., 2008), basic fibroblast growth factor (Akita et al., 2008), transforming growth factor (Cho et al., 2010), and platelet-derived growth factor (Yan et al., 2011). However, costs and side effects restricted the use of these compounds and opened the way for new approaches like the use of

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ABBREVIATIONS: MMP, matrix metalloproteinase; PMN, polymorphonuclear neutrophil; TNF, tumor necrosis factor; sTNF, soluble TNF; IL, interleukin; MIP, macrophage inflammatory protein; M-CSF, macrophage–colony-stimulating factor; GM-CSF, granulocyte macrophage–colony-stimulating factor; G-CSF, granulocyte–colony-stimulating factor; IFN, interferon; SDF-1, stromal cell-derived factor-1; TCA-1, T cell activation-1; TIMP, tissue inhibitor of metalloproteinase.

protectors or co-receptors of these growth factors such as mimetics of endogenous sulfated glycosaminoglycans (Garcia-Filipe et al., 2007; Barbier-Chassefière et al., 2009).

Since antiquity, plant extracts such as St. John's wort (*Hypericum perforatum* L.) have been used to treat wounds within folk medicine in various countries. However, in the majority of cases, the composition and the scientific evidence of their efficiency remain to be established. It is noteworthy that Cicaderma ointment (Boiron, Lyon, France) is marketed mainly in Europe and has been used since the middle of the 20th century in the treatment of wounds, superficial burns of limited extent, and insect bites. This ointment is prepared by the extraction of fresh *Calendula officinalis* L., *H. perforatum* L., and *Achilea millefolium* L. aerial parts in Vaseline mixed with the hydroalcoholic extract of *Ledum palustre* L. Separately, some of these extracts, which are known to inhibit inflammation processes and matrix metalloproteinase (MMP) activity (Oztürk et al., 2007; Dell'Aica et al., 2007a,b; Süntar et al., 2011), are traditionally used to improve wound healing.

To better understand the claimed use of Cicaderma in wound healing and identify new therapeutic targets, we tested its effects in a model of necrotic skin ulcer healing that reproduces human chronic wound (Barbier-Chassefière et al., 2009) and investigated the dynamic processes implicated in wound healing. For the first time, the expression levels of 40 cytokines/molecules involved in the inflammation processes were analyzed when the ulcer reached its maximal area and during wound healing.

Materials and Methods

Animal Model of Skin Ulceration. Housing of animals and anesthesia were performed following the guidelines established by the Institutional Animal Welfare committee with the European guide for care and use of laboratory animals. Standardized skin ulceration was performed by intradermal doxorubicin (Doxorubicin Teva 0.2%) injection on the shaved dorsum of male Swiss mice (Janvier, Le Genest-St-Isle, France) as described previously (Barbier-Chassefière et al., 2009). In brief, animals were anesthetized intraperitoneally with sodium pentobarbital (Céva Santé Animale, Libourne, France). The backs of the mice were shaved with a hair clipper and depilated with Veet depilatory cream (Reckitt Benckiser, Massy, France). Two days after depilation, mice received 150 μl of a 2 mg/ml doxorubicin solution by intradermal injection on the depilated area. The maximum of skin ulcer area was reached 11 days after doxorubicin injection. That day (day 1) was the first day of treatment with Cicaderma or Vaseline (the main excipient of Cicaderma), which were then applied topically every 2 days on the ulcer. Ulcers were photographed every 2 days (days 1, 3, 5, 8, 10, 12, 14, 17, 19, and 21) and cleaned until their complete closure. The lesion size was measured three times by using ImageJ software (http://imagej.nih.gov/ij/) for each ulcer, and the mean was calculated. Biological samples for histological analysis were taken from sacrificed animals on days 1, 3, 5, 10, 17, 19, and 30 after the first application of the ointment, whereas biochemical analysis was done on days 1, 5, and 10.

Histological Studies. Skin samples were fixed in formaldehyde-buffered solution and embedded in paraffin wax. Serial 8-μm sections were prepared. Staining with Masson trichrome was used to study the skin regeneration of five mice in each experimental group (Junqueira et al., 1979). For collagen deposition study, tissue samples were stained with sirius red, and pictures were taken by using a Leitz Laborlux 12 PolS microscope under polarized light. For inflammatory cell studies, sections were prepared as above and stained with May-Grunwald-Giemsa. Polymorphonuclear neutrophil (PMN) blue spots (×250) were counted in three sections per mouse with three mice in each group (Barbier-Chassefière et al., 2009). For blood-vessel density evaluation, paraffin-embedded sections of skin samples on days 3, 5, and 10 after doxorubicin injection were rehydrated by using a decreasing percentage alcohol series as described previously (Barbier-Chassefière et al. 2009). Anti-CD31 primary antibody (1: 25 dilution; BD Biosciences, San Jose, CA) was incubated at room temperature for 2 h and washed in 3% bovine serum albumin/phosphate-buffered saline. Then sections were incubated with a second antibody-fluorescein isothiocyanate (anti-rat IgG; 1:100 dilution; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 2 h at room temperature, and after three washes they were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) (Erba et al., 2011). Specific fluorescence intensity was evaluated by using the TECAN Infinite M1000 plate reader (Tecan, Durham, NC). The blood vessel density corresponded to the fluorescence of a section labeled by CD-31 antibody minus the autofluorescence of this section without CD-31 labeling of three sections per mouse ($n = 3$ mice).

Biochemical Studies. Proteins were extracted from skin sample following the manufacturer's instructions with slight modifications. Skin biopsies were minced and incubated in the sample diluent buffer (Quantibody Mouse Inflammation Array 1; Raybiotech, Norcross, GA) for 1 h at 37°C before homogenization by using a Potter-Elvehjem glass-Teflon homogenizer. The homogenate was centrifuged for 5 min at 13,000*g* to remove debris and insoluble material. Aliquots of the supernatant were assayed for total protein content by the bicinchoninic acid method or stored at −80°C until analysis.

These protein extracts (200 μg/ml) were used with Quantibody Mouse Inflammation Array 1 (Raybiotech) to quantify 40 cytokines in the kinetics of wound healing skin subjected to Vaseline or Cicaderma treatment. The binding of each cytokine on the membrane was revealed by autoradiography and quantified by the Protein Array Analyzer for ImageJ program developed for ImageJ software (http://rsb.info.nih.gov/ij/macros/toolsets/Protein%20Array%20Analyzer.txt). Each assay was performed in duplicate from three mice per experimental group for each day tested.

Statistical Analysis. All results reported are the mean (± S.E.M.) of independent determinations. Differences between the means in two groups were evaluated by using Student's paired *t* test; p values <0.05 were considered significant.

Results

Ulcer Area Studies

Intradermal injection of doxorubicin was reported previously to induce a necrotic skin ulcer, which regenerates spontaneously in mice and constitutes a good model to study the first phases of skin wound healing (Barbier-Chassefière et al., 2009). As shown in Fig. 1, the measurement of the ulcer area indicates that topical applications of Vaseline from days 1 to 21 did not modify the kinetics of ulcer closure compared with untreated skin. However, topical treatment with Cicaderma ointment induced an acceleration of healing compared with the Vaseline-treated group by reducing the surface area of ulcer by 20 to 25% in the first week of treatment. It is noteworthy that Cicaderma application significantly induced the complete closure of ulcer 2 days earlier than what was observed with Vaseline. Macroscopic observations indicated that ulcers treated with Cicaderma appeared less inflamed, less red, and less thick than those treated with Vaseline (Fig. 2).

Histological Studies

Dermal Reconstruction. The effects of Cicaderma application on dermal reconstruction have been assessed by his-

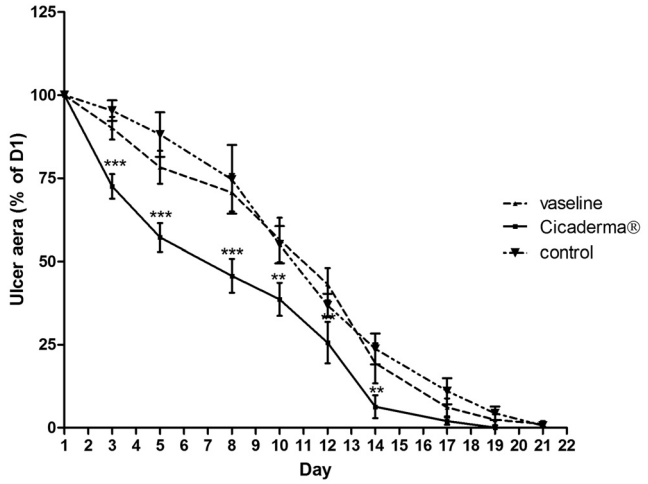

Fig. 1. Effect of Cicaderma on ulcer size. Eleven days after doxorubicin injection (day 0), Vaseline (dashed line) or Cicaderma (solid line) were applied topically to the ulcers every 2 days. The surface area of each ulcer was measured as described under *Materials and Methods* and reported at each time point as the percentage of the surface area at baseline (day 1). Ulcer areas of animals without any treatment are represented by small dotted line. Each result is the mean (± S.E.M.) of three independent determinations in each of 10 mice. Statistical analysis was performed by using Student's paired t test. **, $p < 0.01$; ***, $p < 0.001$.

tological studies using Masson staining throughout ulcer closure. On day 1, corresponding to the maximum of the ulcer area, the total destruction of the epidermis and dermis was clearly observed compared with the mature healthy skin (Fig. 3, a and b). Then the different steps of the healing processes were followed daily. Major modifications including epidermal cell proliferation, illustrated by thickening edges throughout the ulcer, were noticed in both Cicaderma- and Vaseline treated-mice. It is noteworthy that these effects occurred from day 2, just after one topical application of the ointment and were still persistent on day 5 (Fig. 3, c-f). On day 10, the reconstitution of organized skin layers seemed to proceed for the animals treated with Cicaderma, and the thick skin still reflected an important activation (Fig. 3h). In contrast, Vaseline induced the production of new tissue in the dermis and hypodermis, but its organization seemed more anarchic (Fig. 3g), suggesting the development of fibrosis in these animals. These differences between the two treatments were still observed on day 17, mainly in layers below the epidermis that seemed better organized in Cicaderma-treated animals (Fig. 3, i and j). This was confirmed by the organization of collagen (stained in green in Fig. 3), which was less pronounced in sections treated with Vaseline than in those treated with Cicaderma. Finally, at day 29 a greater thickness of the epidermis was seen in Vaseline-treated animals compared with Cicaderma-treated animals (Fig. 3, k and l). Regarding the number of epithelial cell layers, a faster maturation of epidermal structures was induced by Cicaderma. Moreover, the compact and organized dermis in the healing zone exhibited more common features with normal skin than the one observed with Vaseline treatment.

Collagen Organization. The histological studies also allowed us to compare collagen organization in the dermis treated with Vaseline or Cicaderma by sirius red staining, which was visualized by polarized light. The collagen network was studied on the edges of the ulcer at early stages of wound healing (days 1, 3, and 5) or at later stages, inside the healing ulcer area. When the ulcer reached its maximal area (Fig. 4a), collagen fibers displayed heterogeneity and appeared fragmented and with longer and disorganized fibers with horizontal and vertical crossing alongside. This was probably caused by the normal degradation of these fibers by proteases in the ulceration processes. On day 3, collagen was more abundant, without any organization between fibers in Vaseline-treated ulcers (Fig. 4b). In contrast, treatment with Cicaderma induced the presence of better defined intertwining fibers, reflecting the initiation of structural organization of collagen bundles (Fig. 4c). This was confirmed distinctly on days 5 and 10 (Fig. 4, d and f versus e and g). At these times, collagen fibers of Cicaderma-treated ulcers were slim and well defined with clear interlacements that reflected organization of these fibers, whereas the crosswise organization of collagen bundles was not so apparent for Vaseline treatment. When ulcers were finally closed, the collagen organization started to appear in Vaseline-treated animals, whereas in those treated with Cicaderma an increased amount of fibers and a better organization in collagen network were observed (Fig. 4, h and j versus i and k).

Polymorphonuclear Infiltration. Inflammation was evaluated through the measurement of PMN recruitment at the edges of the ulcer (Fig. 5a). The number of PMNs was high when the ulcer reached its maximal area (day 1) and decreased by 2-fold on day 3 after the first treatment with Vaseline. In contrast, the topical application of Cicaderma maintained the high number of PMNs in the wound. On day 5, PMNs increased in the Vaseline-treated group at the level of day 1 and diminished by 30% on day 10. Cicaderma treatment allowed the progressive inhibition of PMN recruitment until day 10, at levels significantly lower than with Vaseline

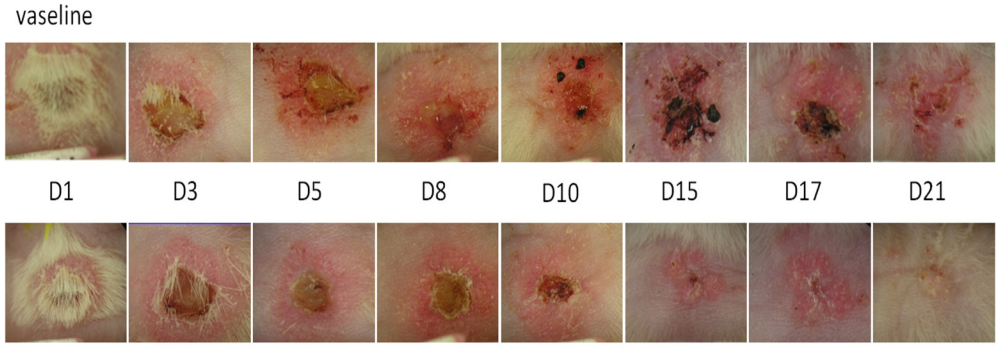

Fig. 2. Macroscopic aspect of ulcer wound healing. Eleven days after doxorubicin injection (day 1), Vaseline or Cicaderma were applied topically to the ulcers every 2 days (D).

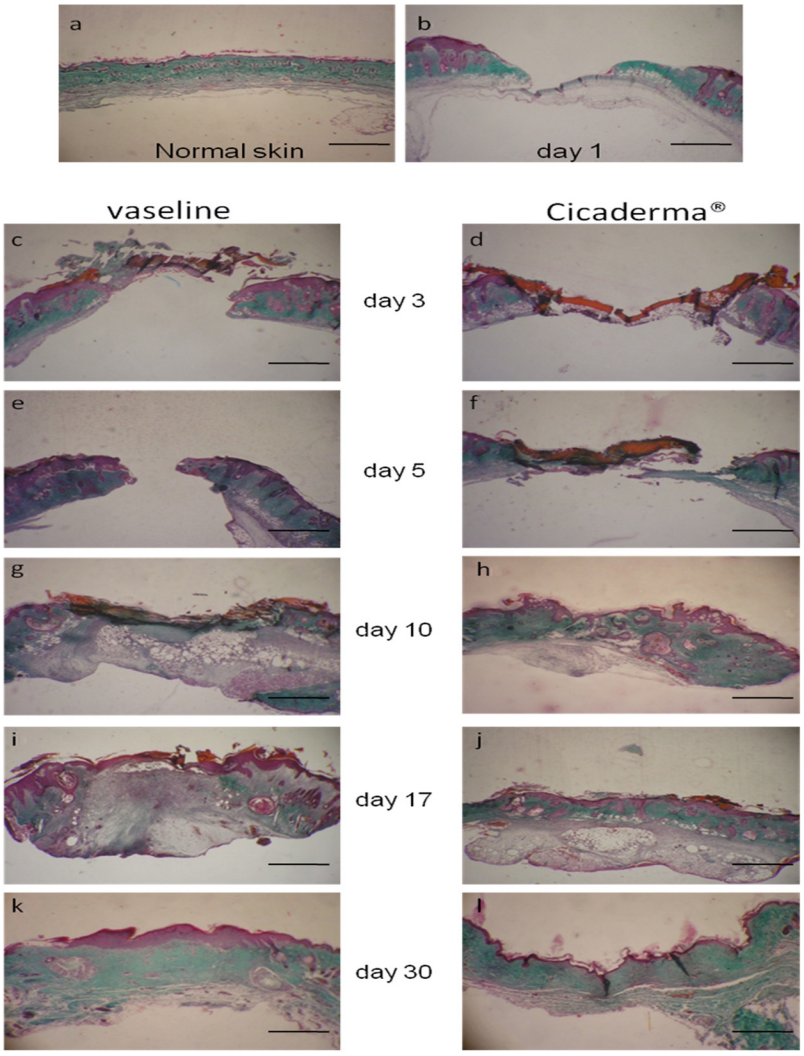

Fig. 3. Histological effects of Vaseline and Cicaderma treatment on skin ulcers. Tissue samples were stained with Masson trichrome as described under *Materials and Methods*. a Mature healthy skin. b, ulcer 11 days after doxorubicin intradermal injection. c, e, g, i, and k, Vaseline-treated ulcer after 2, 4, 9, 16, and 29 days of treatment (D3, D5, D10, D17, and D30), respectively. d, f, h, j, and l, Cicaderma-treated ulcer after 2, 4, 9, 16, and 29 days of treatment (D3, D5, D10, D17, and D30), respectively. Bars, 2.0 mm.

treatment, suggesting a reduction of inflammatory processes in the latter steps of wound healing.

Angiogenesis. CD31 staining was performed to analyze microvessel formation during the angiogenesis phase of wound healing (Vecchi et al., 1994). Blood vessel density was not modified by either treatment, but significantly decreased at day 10 compared with days 3 and 5 (Fig. 5b).

Biochemical Studies

Inflammatory Status at the Maximal Ulcer Area. Before evaluating whether Cicaderma modulated the inflammatory response during the wound healing process, we first measured the level of 40 inflammatory cytokines/molecules in skin ulcer samples when the ulcer reached its maximal area (day 1) and compared it with normal skin (Fig. 6). Eleven days after doxorubicin intradermal injection (day 1), the majority of molecules (34/40) were significantly increased in the nontreated ulcer, except GM-CSF, IFN-γ, IL-12p70, SDF-1, TCA-3, and TIMP-2 whose levels remained unchanged. Just before the first application of the ointment, the TIMP-1 level was remarkably increased by 10 times, whereas B-lymphocyte chemoattractant (BLC), CD30-L, eotaxin, eotaxin-2, Fas-L, fractalkine, G-CSF, IL-3, IL-6, IL-12p40p70, keratinocyte chemoattractant (KC), macrophage inflammatory protein (MIP)-1α, MIP-1γ, sTNF RI, and sTNF RII were augmented by more than 2-fold. Other important molecules involved in wound healing, such as TNF-α, IL-4, and IL-10, rose from 100 to 200%.

Effect of Cicaderma and Vaseline Treatments. Based on these observations obtained on day 1, we compared the levels of all cytokines in ulcers treated with Vaseline or Cicaderma on days 5 and 0 after induction by doxorubicin. Overall, in comparison with Vaseline treatment, the levels of the majority of these cytokines/molecules synthesized in the ulcer were reduced by Cicaderma at days 5 and/or 10.

At day 5 (Fig. 7a), Cicaderma significantly reduced the amount of molecules involved in the TNF pathway such as TNF-α, sTNF RI, and sTNF RII by 24, 43, and 35%, respectively. Moreover, this treatment decreased classic pro-inflammatory cytokines/molecules such as IFN-γ, IL-2, IL-12p40p70, IL-12p70, MIP-1α, and MIP-1γ. We were surprised to find that other known molecules involved in wound healing such as IL-1α, IL-1β, or GM-CSF were not significantly modified by the two treatments (data not shown). It is noteworthy that the amounts of some anti-inflammatory cytokines were also reduced by topical application of the ointment. Cicaderma reduced IL-4 level by 23% and maintained a stable level of IL-10 4 days after the first application while Vaseline increased IL-10 by approximately 40% (Fig. 7a).

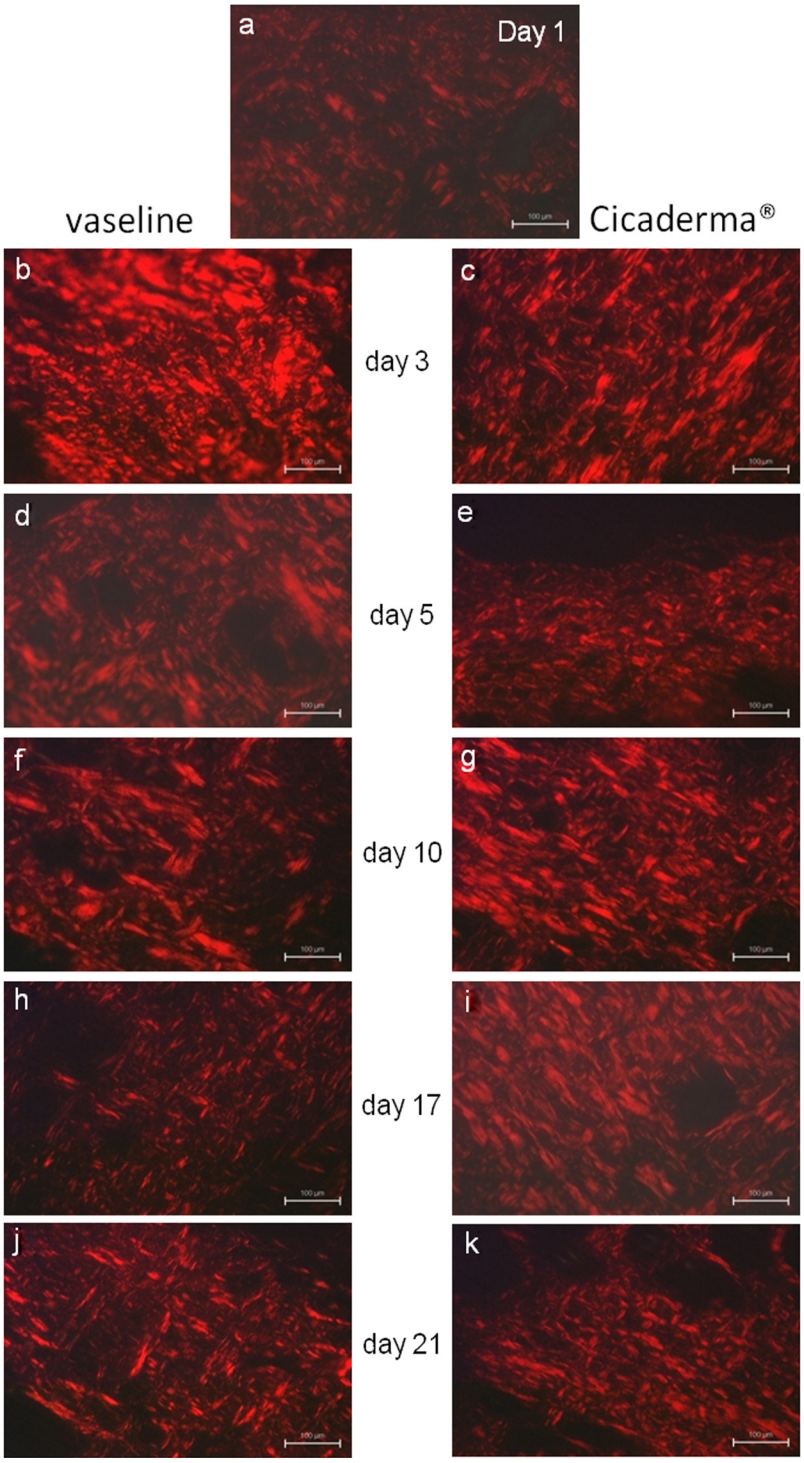

Fig. 4. Histological effects of Vaseline and Cicaderma treatment on skin ulcers. Tissue samples were stained with sirius red as described under *Materials and Methods*. Representative pictures from the area corresponding to the ulcer edges (days 1, 3, and 5) or inside the healing ulcer area at later stages (days 10, 17, and 21) are shown. a, ulcer 11 days after doxorubicin intradermal injection. b, d, f, and h, Vaseline-treated ulcer after 2, 4, 9, and 16 days of treatment (D3, D5, D10, and D17), respectively. c, e, g, and i, Cicaderma-treated ulcer after 2, 4, 9, and 16 days of treatment (D3, D5, D10, and D17), respectively. Bars, 100 μm.

G-CSF and M-CSF, described as hematopoietic molecules, were significantly reduced by Cicaderma, whereas their amount was not influenced by Vaseline compared with day 1 (Fig. 7a). Moreover, the level of the metalloproteinase inhibitors TIMP-1 and TIMP-2 involved in collagens and extracellular matrix degradation were reduced after Cicaderma treatment by 68 and 33%, respectively. Finally, the detection of Fas ligand, which is involved in the regulation of apoptotic cell death, was also diminished by 50% in Cicaderma-treated ulcer compared with the Vaseline group (Fig. 7a).

On day 10, the healing processes continued, and we noticed that there were no statistical differences between the two treatments for the majority of proteins (27/40) (Fig. 7b). However, the expression of 13 cytokines/molecules was significantly decreased by 15 to 35% by Cicaderma compared with Vaseline treatment (Fig. 7b). Cicaderma was able to significantly reduce the level of the proinflammatory cytokines fractalkine, IL-12p70, IL-17, MIP-1α, and TNF-α, but also the anti-inflammatory cytokines (IL-4 and IL-13) or other molecules (MCP-1, M-CSF, lymphotactin, Rantes, SDF-1, and TIMP-2). The amount of proinflammatory cytokines IL-17 and Rantes was

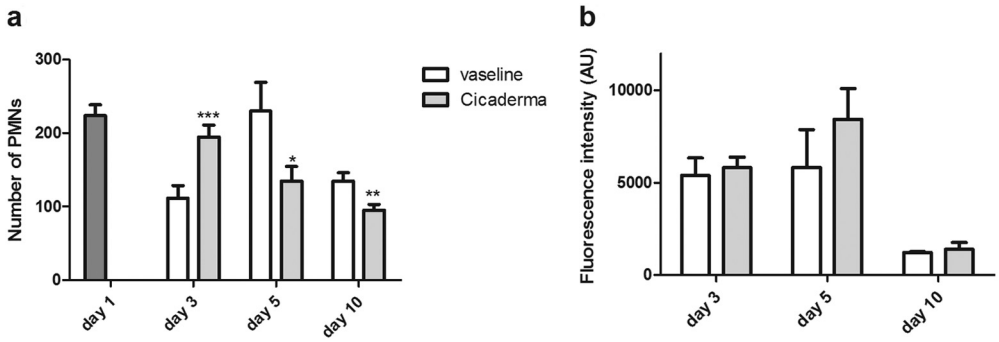

Fig. 5. a, leukocyte recruitment at the ulcer edges. Vaseline and Cicaderma treatment at days 3, 5, and 10 were established after May-Grunwald coloration of paraffin sections. Inflammatory cells were counted in 10 independent fields of three sections of three mice of each experimental group. Results are expressed as the mean ± S.E.M. b, for blood vessel density evaluation, paraffin-embedded sections of skin samples at days 3, 5, and 10 after doxorubicin were used. Specific fluorescence intensity linked to anti-CD31 primary antibody was evaluated by using the TECAN Infinite M1000 plate reader. The blood vessel density corresponded to the fluorescence of a section labeled by CD-31 antibody minus the autofluorescence of this section without CD-31 labeling of three sections from three mice. Results are expressed as the mean ± S.E.M. Statistical analysis was performed by using Student's paired t test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

reduced by 33 and 35%, respectively (Fig. 7b). The hematopoietic molecules MCP-1 and SDF-1 were decreased by 29 and 26%, while the anti-inflammatory cytokine IL-13 was maintained stable close to the level observed on day 1 in Cicaderma-treated ulcer, preventing the increase induced by treatment with Vaseline (Fig. 7b). Lastly, Vaseline application increased TIMP-2 by 25%, whereas Cicaderma reduced the level significantly by 18% (Fig. 7b).

It is noteworthy that five molecules involved in inflammation (IL-12p70, IL-4, M-CSF, MIP-1α, and TNF-α) were significantly diminished on both days 5 and 10 (Fig. 8). Therefore, Cicaderma seemed to modulate the inflammation during wound healing by maintaining a reduced level of these specific factors compared with Vaseline. As presented in the histological studies on day 5 (Figs. 3, e and f and 4, d and e) and day 10 (Figs. 3, g and h and 4, f and g), the regulation of these five molecules probably was implicated in epidermal-cell proliferation, better collagen organization, and quicker re-epithelialization of the skin. As for day 5, TNF-α, the major cytokine involved in inflammation, was maintained stable by Cicaderma treatment, whereas the Vaseline-treated ulcer induced a higher level than at day 1 (Fig. 8a). However, the level of TNF-α with Cicaderma remained higher than in healthy skin. Whereas the levels of M-CSF and IL12p70 in Vaseline-treated ulcer remained unchanged from day 1 and close to those observed in normal skin (Fig. 8, b and c), Cicaderma reduced significantly the concentration of these cytokines at a lower level than on day 1 and in healthy skin. It is noteworthy that Cicaderma reduced the level of IL-4 from the first application at a level close to the one of healthy skin, whereas the IL-4 level remained equivalent to day 1 and higher than in normal skin after Vaseline application (Fig. 8d). The concentration of MIP-1α in Cicaderma-treated ulcer was significantly lower than the one observed with Vaseline treatment but it remained higher than in normal skin (Fig. 8e).

Discussion

Nonhealing wounds remain a major health problem, and new treatments are required to accelerate ulcer closure. Using a mouse model of skin ulcer induced by intradermal

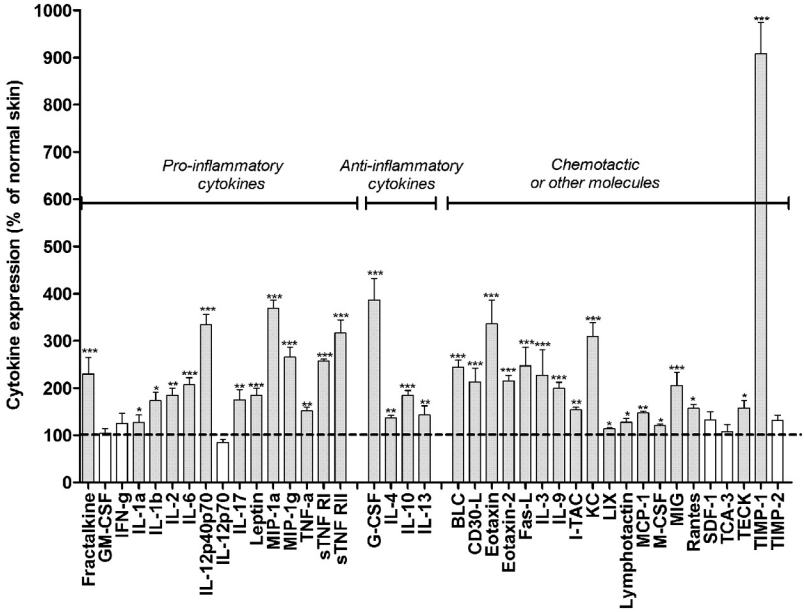

Fig. 6. Cytokine modifications at the maximum of the ulcer area (day 1). Cytokines were extracted as described under *Materials and Methods*, and their quantification was done by using Quantibody Mouse Inflammation Array 1 from Raybiotech. Results are expressed as percentage of cytokine level in normal skin and represent the mean ± S.E.M. of three animals in duplicate. Statistical analysis was performed by using Student's paired t test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

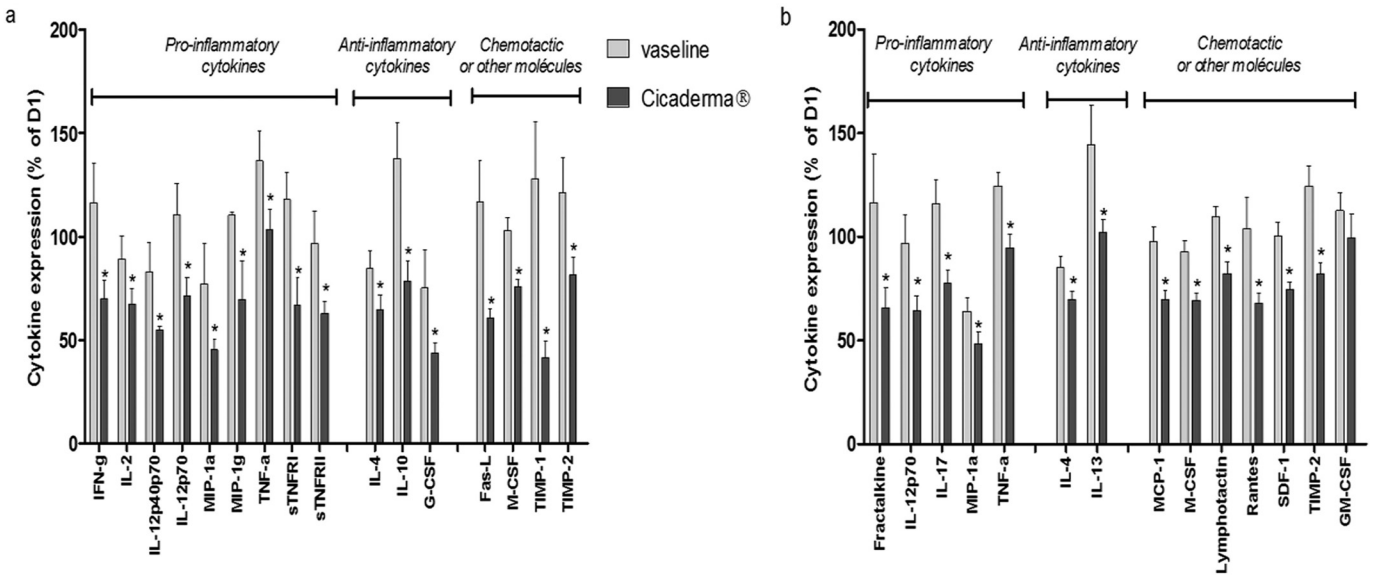

Fig. 7. Cytokine levels after treatment by Vaseline or Cicaderma (days 5 and 10). a, cytokines statistically modified after 4 days (day 5) of Cicaderma treatment. b, GM-CSF level and cytokines statistically modified after 9 days (day 10) of Cicaderma treatment. Cytokines were extracted as described under *Materials and Methods*, and their quantification was done by using Quantibody Mouse Inflammation Array 1 from Raybiotech. Results are expressed as percentage of cytokine level measured at the maximum of the ulcer area (day 1) and represent the mean ± S.E.M. of three animals in duplicate. Statistical analysis was performed by using Student's paired *t* test. *, $p < 0.05$.

injection of doxorubicin (Barbier-Chassefière et al., 2009), the kinetics of the ulcer closure were studied in the presence of Cicaderma ointment. To focus on skin wound healing processes, topic treatment with only Cicaderma started at day 1 when the ulcer reached its maximal area. This treatment is compared with the classic clinical treatment using petroleum jelly (Vaseline), which provides a protective moist environment that facilitates re-epithelialization and wound healing and is classified by the Food and Drug Administration as a skin protectant (Food and Drug Administration, HHS, 2003).

The Hemostasis/Inflammation Phases. Eleven days after doxorubicin injection, the area of the ulcer was maximal. Cell damage, blood vessel injury, and degradation of the collagen network induced by many toxic effects of doxorubicin, including the production of an important oxidative stress, led to clot formation. This clot is considered as an important reservoir of molecules such as cytokines, growth factors that are involved in the chemoattraction of various cells during the early steps of healing (Frank et al., 2000; Marin et al., 2001). In our ulcer model, 11 days after doxorubicin injection, the expression of cytokines/molecules was completely modified compared with healthy skin (Fig. 5). Among the numerous molecules classically involved in the early steps of hemostasis/inflammation phases of wound healing, Rantes, MCP-1, MIP-1α, MIP-1γ, eotaxin, and fractalkine were significantly increased, which correlates with the recruitment of inflammatory cells in the wound bed, as demonstrated by PMN counting (Fig. 5a). PMNs are described as a major source of proinflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α, which are overexpressed in our ulcer model, to stimulate newly attracted monocytes to differentiate into M1 macrophages. These proinflammatory cytokines can also be released by endothelial cells and peripheral blood monocytes in response to thrombin stimulation (Mahdavian Delavary et al., 2011). In addition to these inflammatory cytokines, we noticed a significant 4-fold increase in M-CSF, a hematopoietic cytokine described as an important chemoattractant for PMNs.

Effects of Cicaderma on the Granulation Phase. It is noteworthy that from its first topical applications (days 3 and 5) Cicaderma induced a significant acceleration of ulcer closure as shown by the macroscopic measurement of the ulcer area. The effect of Cicaderma allowed not only the recovery of a mature epidermal structure, a more compact and organized dermis, but also a rapid improvement of the collagen bundle organization close to mature healthy skin. Differences observed in the collagen fiber network can be notably related to *H. perforatum* L., which has been described as an inhibitor of MMP-2 and MMP-9 activities (Dell'Aica et al., 2007a). *H. perforatum* L. down-regulates the expression of both MMPs through the inhibition of the extracellular signal-regulated kinase 1/2 signaling pathway (Donà et al., 2004). In addition, the activation of the fibroblasts and their increased collagen production by *H. perforatum* L. (Oztürk et al., 2007) strengthen the beneficial effects of Cicaderma in the granulation phase of wound healing. *H. perforatum* L. was also described to reduce in vivo the recruitment of PMNs attaching to the vascular endothelium at the site of injury, leading to a diminution of inflammation and angiogenesis (Dell'Aica et al., 2007b). This can be linked to the decrease of PMNs, considered as inflammatory markers, in the wound bed of Cicaderma-treated ulcers. This reduction in the PMN number is not only mirrored by the decrease of hematopoietic cytokines levels such as G-CSF and M-CSF, but also by the reduction of MIP-1α and TNF-α levels, in accordance with the improvement of the granulation phase seen in our histological studies.

According to the central role of TNF-α proposed by Weinstein and Kirsner (2010) in the pathogenesis of wound healing, the reduction in TNF-α level associated with the decrease of the TNF receptors sTNF RI and sTNF RII induced by Cicaderma at day 5 is of importance to explain the accel-

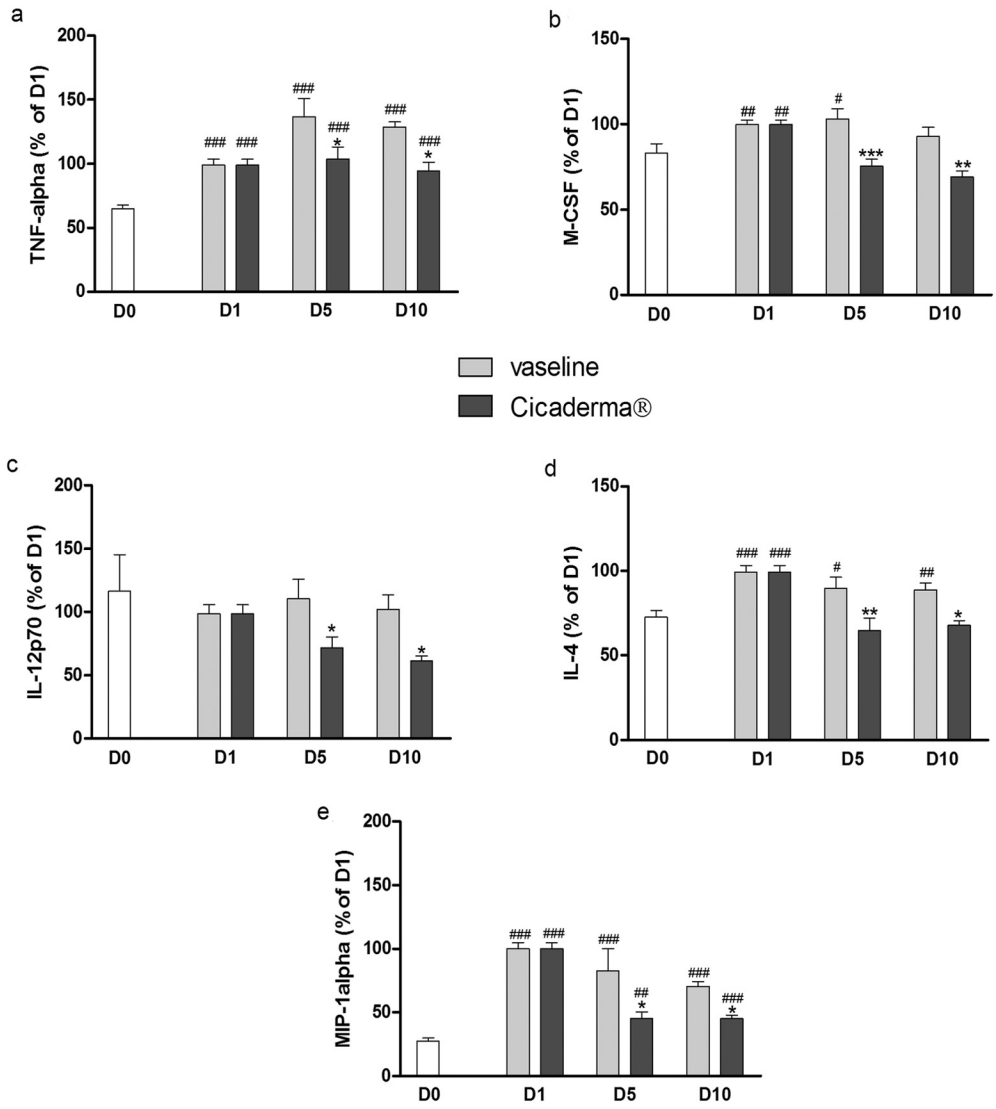

Fig. 8. Levels of TNF-α (a), M-CSF (b), IL-12p70 (c), IL- 4 (d), and MIP-1α (e) in normal skin, at the maximum of ulcer area (D1), 4 days (D5), and 9 days (D10) after the first application of Vaseline or Cicaderma. Cytokines were extracted as described under *Materials and Methods*, and their quantification was done by using Quantibody Mouse Inflammation Array 1 from Raybiotech. Results are expressed in percentage of cytokine level measured at the maximum of ulcer area (day 1) and represent the mean ± S.E.M. of three animals in duplicate. Statistical analysis was performed by using Student's paired *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ Vaseline versus Cicaderma. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with normal skin.

eration of ulcer closure. The decrease of TNF-α could participate in the down-regulation of numerous inflammatory cytokines, i.e., IL-1, IL-6, IL-12, and IL-17, but also the synthesis of cell surface adhesion molecules involved in keratinocyte proliferation, PMN migration, and adhesion to endothelium (Mahdavian Delavary et al., 2011). In the granulation phase, proinflammatory cytokines such as TNF-α and other molecules of the TNF pathway are produced by macrophages/monocytes predominantly recruited by MIP-1α (Kondo and Ishida, 2010). However, the reduction of anti-inflammatory cytokines IL-4 and IL-10 by Cicaderma suggests that this ointment did not simply act as an anti-inflammatory system enhancer but as a general modulator regulating both proinflammatory and anti-inflammatory processes such as TNF-α (Liechty et al., 2000; Werner and Grose, 2003). It is noteworthy that *C. officinalis* L. was able to reduce the levels of TNF-α, IFN-γ, and IL-6 in an in vivo model of inflammation (Preethi et al., 2009) and promote re-epithelialization in a skin excision model (Preethi and Kuttan, 2009). Other studies suggested that the specific lack of endogenous IFN-γ can reduce IL-12 levels, which significantly enhances granulation tissue formation and wound closure (Ishida et al., 2004). Thus, according to these findings, the beneficial and similar effects of Cicaderma from day 5 on the granulation tissue could be attributed to the presence of *C. officinalis* L. in the ointment.

Effects of Cicaderma on the Angiogenesis and Remodeling Phases. In our ulcer model, the angiogenesis phase appeared to begin before day 5 as shown by the labeling of endothelial cells (Fig. 6b). On days 3, 5, and 10, angiogenesis was not modified by Cicaderma treatment, but further studies should be used to confirm this finding. However, Cicaderma modified the pattern of fractalkine, IL-13, IL-17, lymphotactine, Rantes, and SDF-1 only at day 10, whereas only IL-4, IL-12, MCP-1, M-CSF, TIMP-2, and TNF-α were decreased on both days 5 and 10. While highlighting the importance of these cytokines in the regulation of skin wound healing, these findings suggest that 1) the remodeling phase of the wound healing process starts between days 5 and 10, and 2) Cicaderma treatment acts possibly on both the re-epithelialization and remodeling phases. Furthermore, our results show for the first time the involvement in skin wound healing processes of IL-17, an inducer of the production of many other cytokines (IL-6, G-CSF, GM-CSF, IL-1β, transforming growth factor-β, and TNF-α) and chemokines including IL-8 and MCP-1 (Akdis, 2010), by fibroblasts, endothelial

or epithelial cells, keratinocytes, or macrophages. Fractalkine, which is secreted by monocytes/macrophages and endothelial cells in response to inflammatory mediators and oxidative stress, was decreased by Cicaderma treatment. This could reduce macrophage and fibroblast accumulation (Ishida et al., 2008), endothelial cell production of vascular endothelial growth factor (Ryu et al., 2008), and finally angiogenesis (Clover et al., 2011). On day 10, because SDF-1 is proposed to simultaneously promote re-epithelialization and delay contraction, the reduction of SDF-1 level by Cicaderma implies the slowing down of the re-epithelialization phase and the acceleration of wound contraction, which is classically described to improve skin wound healing in rodents (Sarkar et al., 2011). On the contrary, the important decrease of G-CSF level for both treatments suggests the reduction of wound contraction. Thus, Cicaderma could play an important role in the improvement of wound healing kinetics by participating in the fine-tuning of the contraction regulation. Furthermore, Cicaderma did not modify the level of GM-CSF described to be essential for scarless wound repair. The low level of GM-CSF is classically associated with a stronger macrophage infiltration in the wound, with an increase of angiogenesis and better healing (Fang et al., 2010). Thus, the inability of Cicaderma to modify the GM-CSF level in this model while down-regulating a few others (Fig. 7b) may be hypothesized to contribute and promote faster wound healing without keloid scar formation (Yeh et al., 2009). Indeed, the lymphotactin level can be linked to the presence of PMNs, which are the main producers of this cytokine. The reduction of lymphotactin level induced by Cicaderma could be therefore correlated to the diminution of PMNs in the wound bed and would be of particular interest for improving healing and reducing the risk of keloid scar formation. In addition, the slim, defined, and well organized collagen fibers in the Cicaderma-treated group reinforce the improved quality of the final remodeling of the wound, with less inflammation and no keloid scar formation.

In conclusion, this study demonstrated that Cicaderma ointment, a mix of well known natural extracts, modulates inflammation, promotes re-epithelialization, and accelerates skin wound healing in a skin ulcer model in mice (Fig. 9). This ointment can be proposed to accelerate the healing of small wounds, and its potential use in major diseases such as burns and radiodermatitis should be considered. Moreover, our data suggest the possibility it specifically regulates the first steps of wound healing through the modulation of a few cytokines, whose regulation clearly makes a potential target for new pharmacological therapies in chronic wound pathologies. Finally, our data strengthen the emerging concept of a sequential treatment to improve wound healing and involving different pharmacological tools.

Authorship Contributions

Participated in research design: Morin, Caredda, and Courty.

Conducted experiments: Morin, Roumegous, and Barbier-Chassefière.

Performed data analysis: Morin, Roumegous, and Carpentier.

Wrote or contributed to the writing of the manuscript: Morin, Garrigue-Antar, Caredda, and Courty.

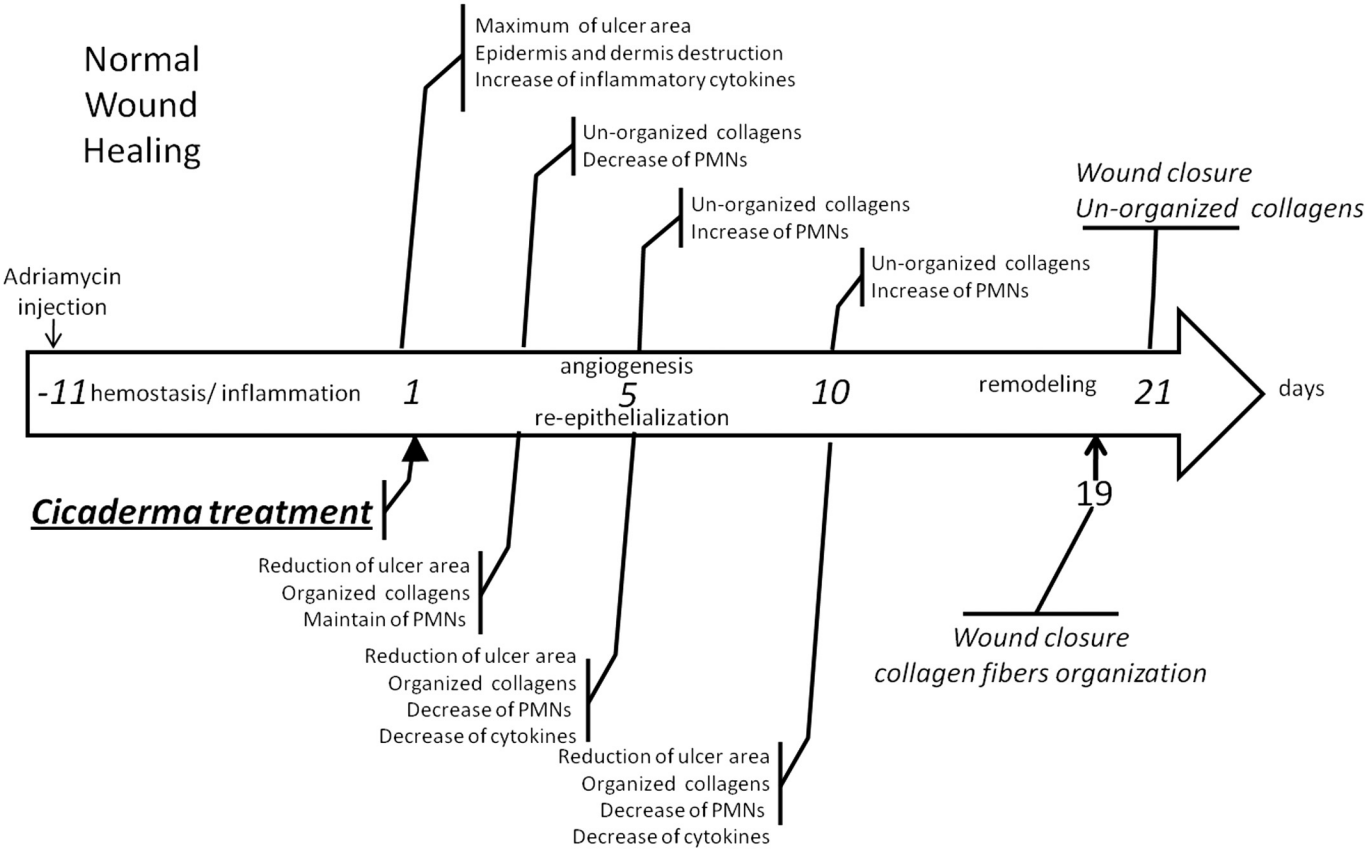

Fig. 9. Sequence of events associated with normal and Cicaderma-treated wound healing.

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