

Skin Wound Healing in Axolotls: A Scarless Process

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ABSTRACT

Urodele amphibians, such as the axolotl (*Ambystoma mexicanum*), have the unique faculty among vertebrates to regenerate lost appendages (limbs and tail) and other body parts (apex of the heart, forebrain and jaw) after amputation. Interestingly, axolotls never seem to form scar tissue at the site of amputation once regeneration is completed. Before now, very few studies were directly focused on the description of the events happening during wound healing after a skin injury in salamanders. In this paper, we directly investigated skin wound healing after excisional wounding which removed the epidermis, dermis and basement membrane in the axolotl. Axolotls were wounded with a 1.5-mm skin biopsy punch. Results show rapid re-epithelialization of the wound within 8 hrs after wounding. Histological analysis of wound healing confirmed the absence of tissue fibrosis throughout the process and shows that skin integrity is re-established by 90 days after wounding. Results also reveal the absence of neutrophils in the wound area, suggestive of a lack of or low inflammatory response. The expression of proteins central to wound healing seemed different than in mammals as α -smooth muscle actin was absent and transforming growth factor β -1 was only transiently expressed during wound healing in the axolotl. Finally, subcutaneous injections of bleomycin were performed to verify whether the induction of scar tissue was possible in axolotls. Surprisingly, results show that axolotls are not resistant to bleomycin-induced tissue fibrosis, but the resulting scar tissue does not seem to contain significant amounts of collagen.

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Urodele amphibians, such as the axolotl (*Ambystoma mexicanum*), are best known for their unequalled ability among vertebrates to regenerate amputated body parts. Over the years, tissue regeneration has mostly been characterized through the study of limb, lens, tail, and spinal cord regeneration in these animals. At the end of the process, regeneration usually leads to the complete restoration of structure and function of the damaged limb or organ. The regeneration process observed in salamanders comprises three essential steps: (1) wound healing; (2) the generation of a population of undifferentiated cells which give rise to a blastema; and (3) a phase of redevelopment in which blastema cells will proliferate to regenerate the lost tissue. The first phase of regeneration in urodeles (wound healing+blastema formation) shares many similarities with mammalian wound healing (Roy and Lévesque, 2006), although mammals cannot regenerate amputated appendages and form scars after healing their wounds. Interestingly, salamanders never seem to form any scars between the stump and the newly regenerated limb or tail.

In humans and most mammals, a superficial injury of the skin will heal rapidly and will not leave any residual defect. On the

contrary, a deep skin injury (i.e. that goes through the epidermis to reach and damage the dermis) will cause a series of inter-related events leading to the formation of a scar once wound healing is completed. Classically, mammalian wound healing is divided in three steps: (1) inflammation; (2) new tissue formation; and (3) remodeling (Gurtner et al., 2008). The inflammation phase of wound healing begins immediately when damage occurs to the skin. Hemostasis is one of the earliest mechanisms to happen and re-establishes the barrier between the body and the environment. In the following minutes and hours, immune cells such as

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neutrophils and macrophages invade the wounded area to clear bacteria and cellular debris (Martin, '97; Gurtner et al., 2008). As this clearing process goes on, inflammation will mainly be caused by the release of cytokines (e.g. epidermal growth factor, transforming growth factor-beta (TGF- β), tumor necrosis factor-alpha, and interleukins alpha or beta) by the adjacent extracellular matrix (ECM) and immune cells. These cytokines direct the outcome of wound healing by influencing immune cell response, ECM production, and skin cells movement and proliferation. The second phase of wound healing in mammals is characterized by the formation of new tissues. It is during this phase that keratinocytes migrate under the scab to re-establish the epidermal layer. In addition, some fibroblasts around the wounded area transform into myofibroblasts, characterized by the expression of alpha-smooth muscle actin (α -SMA) and migrate in the wound (Desmouliere et al., 2005). These myofibroblasts play a very important role in mammalian wound healing. Using their smooth muscle-like properties, they contract to help the wound to close. Unfortunately, these cells are also the ones responsible for the appearance of the scar as they secrete large amounts of collagen (Martin, '97; Desmouliere et al., 2005; Gurtner et al., 2008). Collagen fibers secreted by myofibroblasts accumulate in small parallel bundles that causes the scar (Ferguson and O'Kane, 2004). In humans, this phenomenon is enhanced in patients suffering from systemic sclerosis disease and often in patients who suffered burn injuries (Wynn, 2008). The last phase of wound healing consists of the remodeling of the wounded area. Most of the immune cells and myofibroblasts implicated during the first two phases of wound healing will die by apoptosis during this final phase. Finally, during the following weeks and months, extracellular proteins such as matrix metalloproteinases will rearrange the collagen fibers in the scar tissue (Gurtner et al., 2008). Nowadays, many avenues are studied to develop new treatments that could improve skin wound healing in humans. For example, numerous research groups aim at improving the scar appearance by altering specific signaling pathways involving proteins, such as TGF- β s, connexins, or osteopontin, during wound healing (Mori et al., 2006, 2008; Ferguson et al., 2009). Tissue-engineered skin derived from allogenic or autologous cells is also frequently used on patients who suffered from burns or chronic wounds (MacNeil, 2007). Although these avenues already promise to improve wound healing in humans, it is important to consider other research fields in case progression toward the establishment of new therapies doesn't go as fast or as far as expected. The study of regeneration in urodele amphibians provides us such an opportunity to directly examine *perfect* wound healing of the skin in a vertebrate model organism.

Most of what is known about wound healing in axolotls and newts was characterized by studying limb regeneration. After amputation, epidermal cells (i.e. keratinocytes) surrounding the open wound rapidly migrate on the wound bed using pseudo-

podial projections (Repesh and Oberpriller, '78; Wallace, '81). It was shown that these cells rarely divide while migrating to cover the amputation plane (Chalkley, '54; Hay and Fischman, '61). This wound healing process is rapid and can even be completed in less than 2 hr in limbs of small axolotls (Carlson et al., '98; Levesque et al., 2007). This step is critical for regeneration to proceed, as preventing wound epidermis formation by different approaches blocks limb regeneration (Wallace, '81). This brought many to view the wound epidermis as a permissive tissue for regeneration. Surprisingly, very few papers were published with results directly related to the description of skin wound healing in salamanders. One of the first to conduct a descriptive study of the process in the axolotl was Ziegels in 1971. After inducing 5 mm excisional wounds on axolotl tails, he studied the movements of melanocytes in the healing skin. He showed that melanocytes take between 48 and 72 hr after wounding to migrate and repopulate the skin in the wounded region (Ziegels, '71). Among his observations was the fact that wounds were totally covered by epithelial cells within the first 48 hr (Ziegels, '71). He also noted that very few inflammatory cells were present at the wound site in axolotl skin (Ziegels, '71). One of the other few scientists to have studied skin wound healing in salamanders is Donald J. Donaldson. In numerous papers, he has evaluated the migratory characteristics of salamander epidermal cells, using both in vitro and in vivo experiments. Among his discoveries is the fact that newt keratinocytes constitutively express β 1-integrin, which allows them to rapidly migrate over the wound bed (Donaldson et al., '95). Although the publications of Ziegels and Donaldson bring interesting data on salamander wound healing, they lack a thorough histological analysis of tissue movement in the first hours after wounding. Current methods now also allow for gene and protein expression analysis during the process, which were not available then.

Since salamander regeneration is often stated as being *scarless* and because many mechanisms are common to mammalian wound healing during the initial phase of urodele regeneration, we initiated the characterization of skin wound healing in axolotls. In this paper, we report for the first time the histological characterization of skin re-epithelialization after excisional wounding of axolotl skin. Our data cover the first 90 days of axolotl wound healing with specific emphasis on early events. As others have shown, we observed rapid wound closure of axolotl wounds in less than a day. The results obtained also led us to conclude that the healing of excisional wounds in axolotl shows very little inflammation and never leads to scar formation. Moreover, our data shows that TGF- β 1 and α -SMA are differentially regulated in axolotl wound healing when compared with mammals. Finally, subcutaneous injections of bleomycin in axolotls led to the discovery that these urodeles are not resistant to chemically induced scarring.

MATERIALS AND METHODS

Animal Maintenance and Wounding

Axolotl (*Ambystoma mexicanum*) larvae were purchased from the Ambystoma Genetic Stock Center (Lexington, KY). Animals were maintained in 20% Holtfreter's solution at 19–22°C on a 12-hr of light, 12-hr of darkness cycle. Before wounding, animals were anesthetized in 0.1% MS-222 (ethyl 3-aminobenzoate methane-sulfonate salt; Sigma-Aldrich, St. Louis, MO). Animals used were measured between 7 and 10 cm and were inflicted excisional skin wounds on the right side of their tail. A dermal biopsy punch (Miltex Inc., York, PA) with a diameter of 1.5 mm was used to inflict the wound. After wounding, the epidermis at the center of the 1.5-mm circle was carefully removed with fine tweezers before the animal was placed back in clean Holtfreter's solution. To determine the speed of wound closure, drops of the vital dye, Fast green, were added on the wounds for 1 min and then rinsed with Holtfreter's. Wounds were considered being "open" if green staining remained. For each histology/immuno-histochemistry time point, the animal was anesthetized and tail amputation was performed using a sterilized sharp razor blade. Each sample was fixed overnight in 4% paraformaldehyde in $0.7 \times$ PBS at 4°C ($0.7 \times$ PBS is used for axolotl tissues). The samples were then rinsed, dehydrated, and put in paraffin the next day. Sectioning was performed at a thickness of 10 μ m. Animal care and experiments were carried out in accordance with the Université de Montréal animal care committee's guidelines.

Histology

Before staining, slides were deparaffinized through three baths of toluene for 5 min each. Slides were then rehydrated in a graded series of 100, 90, 70, and 50% ethanol and then with distilled water for 5 min each. Masson's trichrome staining method was used to stain cell cytoplasm in red, nuclei in black, and collagen in blue (Luna et al., '68). Sirius red staining was used to specifically stain collagen fibers (protocol of John A. Kiernan on www.ihcworld.com). To permit the visualization of collagen-specific staining, slides stained with Sirius red were visualized using a microscope equipped with a polarized light. After staining, slides were serially dehydrated for 2×2 min each in 90% EtOH, 100% EtOH, and 100% Xylene. Slides were mounted with Permount (Fisher scientific, Ottawa, ON, Canada) and a minimum of three samples was stained for each time-point.

Assessment of Inflammation During Wound Healing

To measure the infiltration of axolotl skin wounds by neutrophils, the Naphthol AS-D chloroacetate esterase kit (cat. no. 91C-1KT, Sigma-Aldrich) was used. Manufacturer's instructions were followed which gave a bright pink staining for neutrophils and a dark purple tissue staining resulting from counter-staining with hematoxylin solution Gill no. 3.

Immuno-Histochemistry

Tissue sections were deparaffinized and rehydrated as for histology. The ABC staining system from Santa Cruz biotechnologies was used for protein detection (ABC staining system for mouse (cat. no. sc-2017) and rabbit (cat. no. sc-2018) primary antibodies, Santa Cruz biotechnology inc., Santa Cruz, CA). The experiment was performed following the manufacturer's protocol as summarized here: after rehydration, treat with 3% H_2O_2 in $0.7 \times$ PBS 10 min, rinse with $0.7 \times$ PBS 2×5 min, block for 1 hr in 1.5% serum solution diluted in $0.7 \times$ PBS, incubate 30 min at room temperature with primary antibody diluted 1:500 in blocking solution (anti-TGF- $\beta 1$ (cat. no. sc-146), Santa Cruz biotechnology inc., anti- α -SMA (cat. no. MS-133-P0), Neomarkers, Fremont CA), rinse with $0.7 \times$ PBS 3×5 min, incubate 30 min with biotinylated secondary antibody and rinse with $0.7 \times$ PBS 3×5 min, incubate 30 min with AB reagent (avidin + biotinylated horse radish peroxidase) and rinse with $0.7 \times$ PBS 3×5 min, incubate with DAB for about 5 min and rinse in deionized H_2O for 5 min, dehydrate and mount with Permount (Fisher scientific) as for histology.

Bleomycin Injections

Bleomycin (Bioshop Canada inc., Burlington, ON, Canada) was diluted in distilled water to a concentration of 5 mg/mL. During the experiment, an aliquot of the stock was kept at 4°C in a glass tube. Bleomycin is good for up to 3 months at 4°C when kept in a glass container. A protocol was established in order to repeat the experiment originally performed by Yamamoto et al. in the mouse (Yamamoto et al., '99) to allow easier comparisons. A group of axolotls injected with bleomycin were not wounded and a second group were wounded just before the first bleomycin injections. Bleomycin was injected subcutaneously in axolotls using a 10- μ L Hamilton syringe (Hamilton company, Reno, NV). Bleomycin was injected at two concentrations: 1 mg/mL and 100 μ g/mL diluted from the stock in $0.7 \times$ PBS with 0.02% Fast green added to help visualize the injection. Injections of 1 μ L were made on one side of the tail every other day during 30 days. Before the experiment started, two black spots separated by 1 cm were made under the axolotl skin by injecting India ink. These ink spots allowed the injections to be made in the same region of the axolotl body every time to ascertain the effect would be localized. Three control animals (injected with PBS and Fast green only) and eight experimental animals (four injected with Bleomycin at 1 mg/mL and four injected at 100 μ g/mL) were used for the injection experiment without wounding and all survived. For the bleomycin treatment of animals with excisional wounds, four animals per group were used (four control injected with PBS, four injected with 100 μ g/mL, and four injected with 1 mg/mL of bleomycin). Tails of the animals were amputated the day following the last bleomycin injection then fixed and processed as mentioned above.

RESULTS

Healing of Excisional Wounds is Completed in 8 hr in Axolotls

To begin our study, excisional wounds were made on one side of the tail of the axolotl. To do so, a 1.5-mm standard dermal biopsy punch was used to wound the animal. Skin delineated by the biopsy punch was carefully removed without damaging the muscles. Visual examination of the re-epithelialization process was performed to find out how fast the axolotl epidermis would cover the wound bed. To help in the visualization of the process, fast green was added to the wound area immediately after wounding (0h, Fig. 1A and B) and at 1 h (Fig. 1C and D), 4 h (Fig. 1E and F) and 8 h (Fig. 1G and H) after wounding. Fast green is a vital dye that can stain damaged and dead cells; therefore, it can be used to identify exposed cells in the wound bed (Weisenthal et al., '83). Theoretically, there should be no green staining in the wound area when the wound is totally covered by epidermal cells. Immediately after wounding, fast green stained the entire wound area delineated by a dotted line (Fig. 1B). An hour after wounding, addition of fast green stained the entire wound area, although staining seemed to be diminished on the edge of the wound (Fig. 1D). Four hours after wounding, fast green only stained the central region of the wounded area (Fig. 1F), showing the wound healing process was about half completed by that time. The absence of fast green staining suggests that the wound epidermis had completely covered the wound area by 8 hr after wounding (Fig. 1H).

Skin Integrity is Restored in 90 days After Excisional Wounding in Axolotl

After determining that wound closure is completed in about 8 hr in axolotl, a histological analysis of wound healing was done in order to facilitate the description of the process at the cellular level. Masson's trichrome staining was used so that skin and muscle cells would be stained in red and extracellular collagen in blue. The analysis covered a period of 90 days (Fig. 2). Figure 2A shows that the method used for wounding allowed the complete removal of axolotl skin, including the basement membrane, without damaging the underlying muscles. One hour after wounding, some epidermal cells at the edge of the wound are already positioning themselves in preparation for migration over the wound bed (Fig. 2B, inset). Four hours after wounding, epidermal cells have already migrated to cover about half the distance to the center of the wounded area (Fig. 2C, inset). In every sample analyzed, a large portion of this migrating wound epidermis is not tightly attached to the underlying tissue. Results obtained in Figure 1 using fast green and showing complete wound closure by 8 hr after wounding are confirmed by histological analysis (Fig. 2D). At this moment, the wound epidermis has covered the wound bed but is still not lying directly on the muscle (Fig. 2D). Wound epidermis architecture is already starting to share some similarity to that of unwounded epidermis 8 hr after wounding with the presence of

gland cells (large round cells with a big cytoplasm that often looks granular in the epidermis) and other epidermal cells. Over time, the space between the wound epidermis and muscle diminishes so that, a full day after wounding, the skin is in direct contact with the muscle underneath (Fig. 2E). The second part of the histological analysis was performed to determine when the basement membrane made of collagen (stained blue), which lies just under the epidermis, would be regenerated. Forty-eight hours after wounding, some square-shaped cells seemed to appear in the basal epidermis covering the wound (Fig. 2F, white arrowhead in inset). These cells appeared in larger numbers 4 and 9 days after wounding, aligned in one layer close to the muscles (Fig. 2G and H, white arrowhead in inset). The role of these cells was not directly assessed in this study but they are reminiscent of columnar cells observed in the wound epidermis covering the blastema at the late bud stage, during axolotl limb regeneration (Christensen and Tassava, 2000). Nine days after wounding, there is still no sign of basement membrane regeneration in the wound area (Fig. 2H, inset). Many dermal cells accumulate under the epidermis 9 and 12 days after wounding (Fig. 2H and I, blue arrowhead in inset in I) as described previously by Endo et al. in the accessory limb model where dermal cell migration was determined (Endo et al., 2004). Small patches of blue can be observed under the epidermis at 12 and 18 days after wounding, indicating the presence of collagen (Fig. 2I and J). This signifies that the basement membrane is not totally restored 18 days after wounding. The basement membrane is still not completely restored 45 days after wounding, as it shows a fibrous and multi-layered organization (Fig. 2K, inset). Three months after wounding, the integrity of the basement membrane (i.e. structure and blue color) is back to normal. It is impossible to distinguish the wounded area from normal skin 90 days after wounding meaning skin, including the basement membrane, is totally regenerated without scar formation, after wound healing in the axolotl (Fig. 2L).

Very Few Neutrophils are Detected in Skin Wounds of Axolotl

The presence of neutrophils in the wound bed is a very good indicator of an activated immune response in the first hours after wounding. In mammals, neutrophils are detected in the wound just minutes after the injury has occurred and usually stay in the wound area for a few days. They play many important roles from ingesting invading bacteria to secreting pro-inflammatory cytokines (Martin, '97). The detection of neutrophils was assessed as early as 30 min after injury in axolotls, so that initial events would not be missed (Fig. 3A). There were no neutrophils detected in the wound bed of all injured animals during the first day after wounding (Fig. 3A–D). Two days after wounding, a few neutrophils (bright pink cells, green arrowhead Fig. 3F) were found just underneath the newly restored epidermis in some animals (2 out of 4). There were no neutrophils detected four days after wounding (Fig. 3G). In the literature, urodele limb regeneration was previously associated with a downgraded

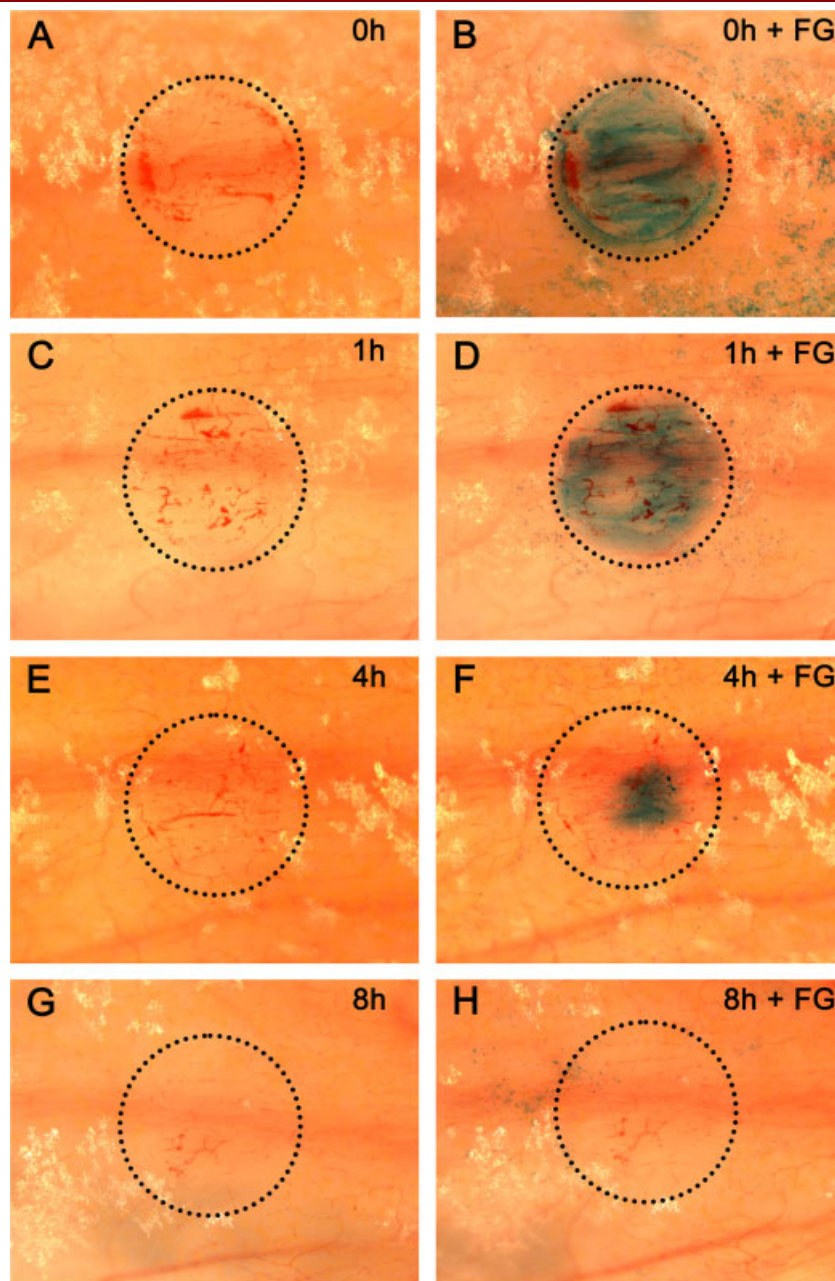


Figure 1. Visual assessment of excisional wound closure in axolotl. Pictures of excisional wounds made in the axolotl skin taken at the time of wounding (A and B), 1 h (C and D), 4 h (E and F) and 8 h after wounding (G and H). Dotted line delineates the wound area. At every time point, wounds were stained with Fast green to visualize the progress of re-epithelialization (B, D, F, and H). It is possible to notice that the edges of the wound are not stained by Fast green 1 h after wounding meaning wound closure has already begun (D). Only about one quarter of the wound area is still uncovered by the migrating epidermis 4 h after wounding (F). The wound is totally covered by the wound epidermis by 8 h after wounding (H). [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

immune response (Sicard, '83; Mescher and Neff, 2006). As a control to the detection of neutrophils during axolotl wound healing, the presence of neutrophils was also assessed during axolotl limb regeneration. In concordance with the literature, it

was not possible to detect any neutrophils at the tip of an amputated limb, 48 hr post-amputation (Fig. 3H). On the other hand, a stabbing wound with deep penetration in the tail muscle was infiltrated by a large number of neutrophils 48 hr after

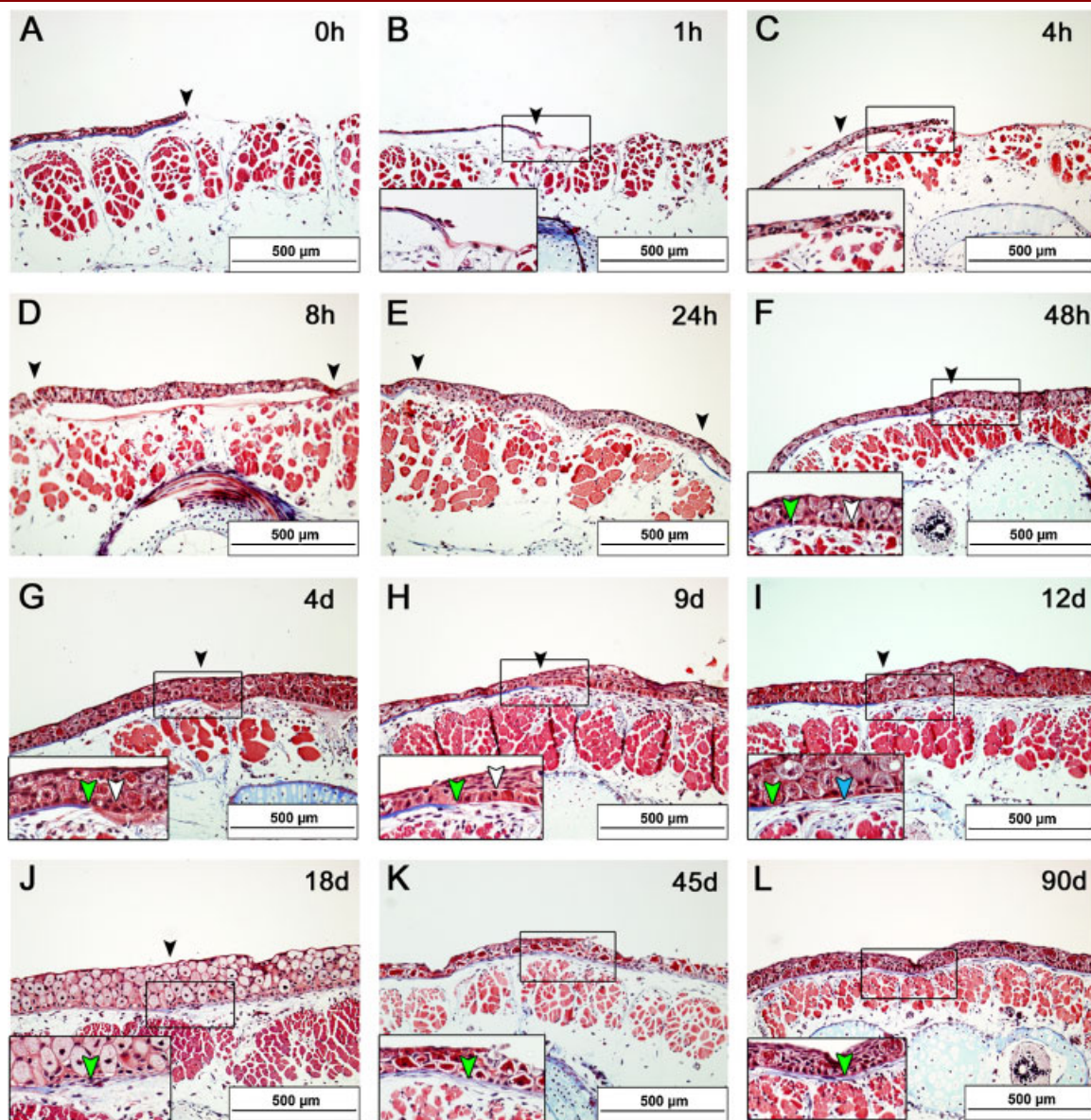


Figure 2. Histological analysis of skin wound healing in axolotl. Skin samples were stained with Masson's trichrome. In each picture, the black arrowhead indicates the edge of the wound with the wound on the right. After wounding, only the epidermal layer was removed without damaging the muscle (A). One hour after wounding, epidermal cells begin to migrate over the wound bed (B, black arrowhead). Four hours after wounding, the migrating epidermis has already covered a significant portion of the wound area (C). Wound closure is completed 8 h after wounding but the wound epidermis is not in direct contact with the underlying muscles (D). One day after wounding, the wound epidermis is now lying directly on the muscles (E). Columnar cells are present in the basal layer of the epidermis 48 h (F), 4 days (G) and 9 days (H) after wounding (white arrowheads). Nine days after wounding, the basement membrane of the skin is still not regenerated (H). Traces of collagen (blue filaments) are observed under the epidermis 12 days (I) after wounding, indicating the basement membrane is partially regenerated. The basement membrane is still not completely regenerated by 18 days (J). The basement membrane is almost completely restored, after 45 days, but has a fibrous aspect (K). 90 days after wounding, skin integrity was restored in every wounded animal and the basement membrane was completely regenerated (L). Green arrowheads identify the collagenous basement membrane of the epidermis (stained in blue) and the blue arrowhead identifies dermal cells. [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

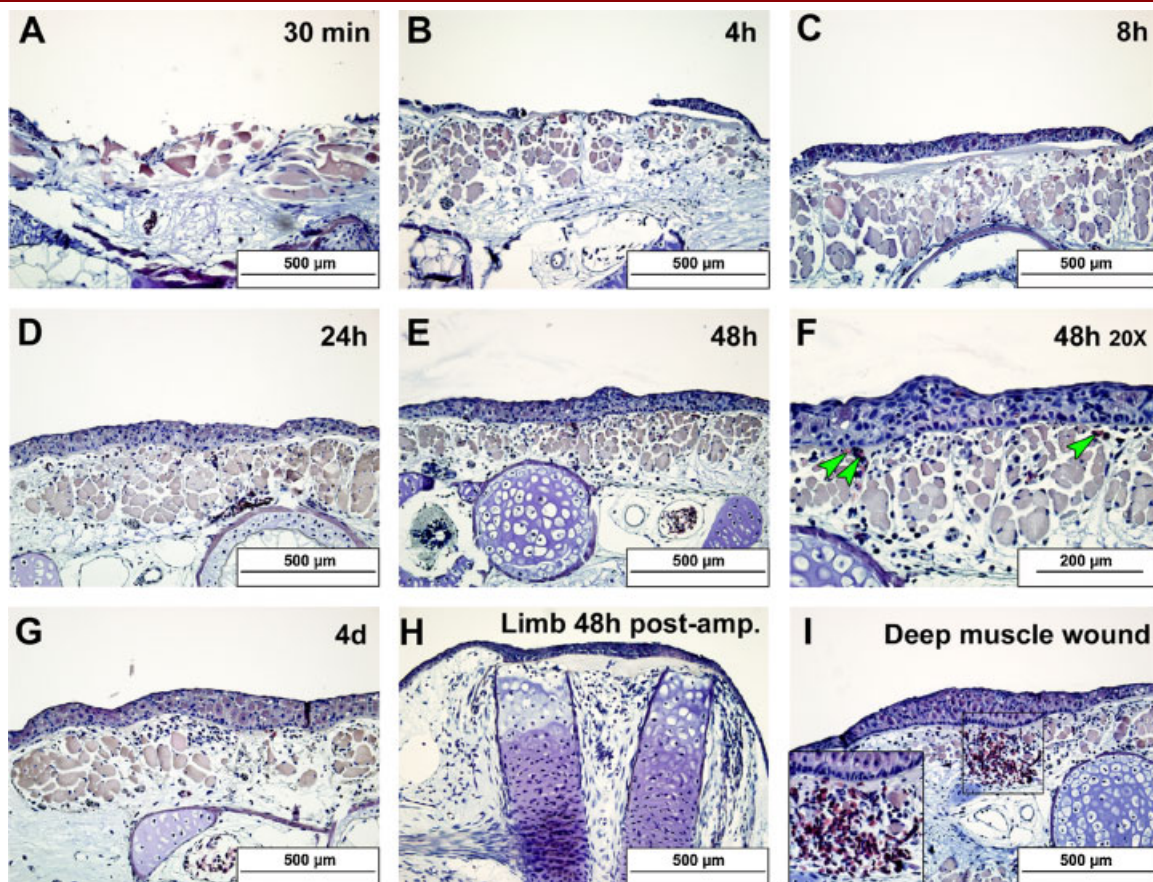


Figure 3. Detection of neutrophils during skin wound healing in axolotl. It was not possible to detect any neutrophil during the first day after excisional wounding of the skin in axolotl (A, B, C, and D). Few neutrophils were found under the healed epidermis 48 h after wounding in some of the animals (E, bright pink cells identified by green arrowhead in F). No neutrophils could be detected in the wounded area 4 days after wounding (G). During axolotl limb regeneration, there are no neutrophils detected at the tip of the amputated limb, 48 h post-amputation (H). A stab wound deep in the muscles can induce many neutrophils to infiltrate the wounded area, 48 h after wounding (I). [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

wounding (Fig. 3I). This latter recruitment of neutrophils at the stab wound site can be attributed to the nature of the wound itself. In a stab wound, contrary to excisional skin wounds or limb amputation, damaged cells and/or tissues are not removed. The damaged tissue is left in place and therefore neutrophils are recruited to the site to help get rid of the cellular debris which are pro-inflammatory.

Skin Injury Does not Lead to Tissue Fibrosis in Axolotl

It is recognized that axolotl never form scars after limb or tail amputation and our present results also demonstrate it for skin wounds. In mammals, expression of α -SMA by myofibroblasts is a good marker of fibrosis during the early events of wound healing (Hinz, 2007; Wynn, 2008). In this study, the presence of α -SMA was assessed in axolotl skin wounds by immunohistochemistry. One hour after wounding, it was impossible to

detect any expression of α -SMA (Fig. 4A). As wound closure progresses, 4 hr after wounding, there is still no production of α -SMA in the wound bed (Fig. 4B). However, intense staining was detected in the walls of the dorsal aorta (Fig. 4B, black arrowhead), which served as an internal positive control (Fig. 4D, inset). A day after wounding, no clear signal could be detected above background level (Fig. 4C). Again, four days after wounding, some skin cells seemed to weakly express α -SMA, but this expression is considerably low compared with the one found in the dorsal aorta (Fig. 4D and inset). Twelve days after wounding, there seems to be no α -SMA in the healed skin of the axolotl (Fig. 4E). As a control experiment, expression of α -SMA was also assessed in the walls of the small intestine, which contains smooth muscles, and gave a very strong signal indicating good cross-reactivity of the antibody used with the axolotl α -SMA protein (Fig. 4F).

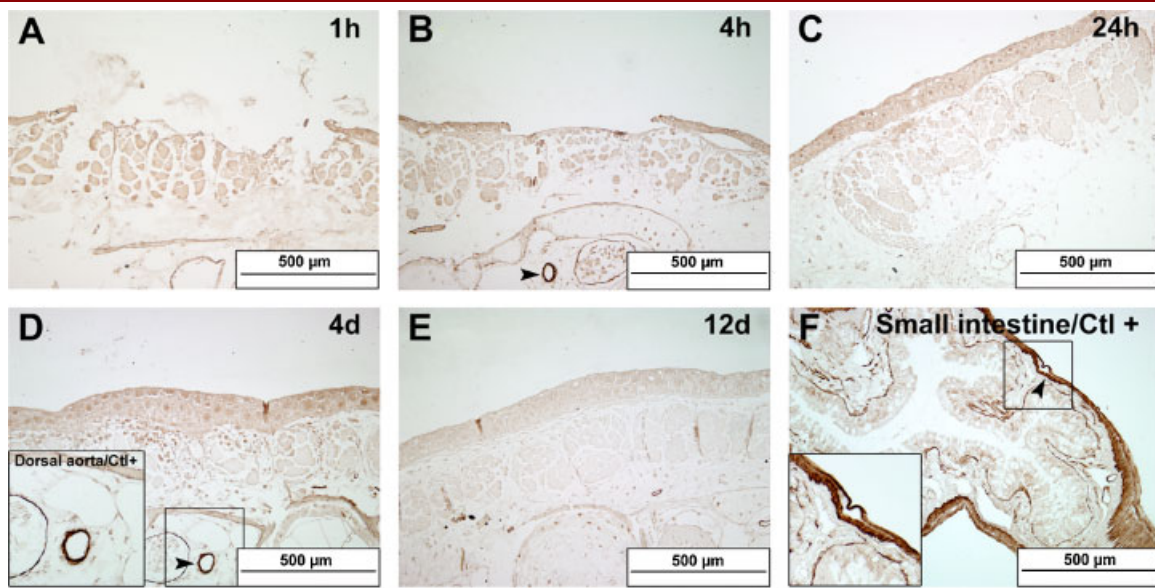


Figure 4. Alpha-smooth muscle actin protein expression during wound healing in the axolotl. Immuno-histochemistry experiment showing that no specific signal could be detected for α -smooth muscle actin in the wounded tissue 1 h (A), 4 h (B), 24 h (C), 4 days (D) and 12 days (E) after wounding. In comparison, strong protein expression was detected in the dorsal aorta (arrowhead in B, arrowhead and inset in D). As a control, protein expression was assessed in sections of axolotl small intestine. Intense protein expression can be seen in intestinal wall (F, inset). [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

Transforming Growth Factor- β 1 is Expressed During Wound Healing in the Axolotl

It is now generally accepted that TGF- β 1 is a central regulator of wound healing (Klass et al., 2009). In a previous study, we showed that TGF- β 1 is expressed in the blastema during limb regeneration in the axolotl (Levesque et al., 2007). In this paper, immuno-histochemistry experiments permitted to detect the production of TGF- β 1 as early as 1 hr after wounding (Fig. 5A). The signal detected is localized to the epidermis surrounding the wound area (Fig. 5A arrowheads and inset). When the wound bed is about half-covered by the epidermis, 4 hr after wounding, TGF- β 1 is still found in the migrating epidermis with a distinct presence around cells of the migrating front (Fig. 5B, inset). Twenty-four hours after wounding, a consistent signal suggesting the presence of TGF- β 1 can be seen in some epidermal cells and underlying ECM in the wound area (Fig. 5C). Four days after injury, very few skin cells show TGF- β 1 expression and the signal coming from the underlying ECM disappears (Fig. 5D). Twelve days after wounding, it is not possible to distinguish a specific signal from background (Fig. 5E). As a control, TGF- β 1 production in an axolotl regenerating limb at early bud blastema stage was measured and specific signal can be observed in blastema cells as previously shown (Levesque et al., 2007) (Fig. 5F).

The Axolotl is Not Resistant to Fibrosis Induced by Bleomycin

Results obtained in this study, combined with the results of other studies, show that the axolotl does not develop fibrosis after skin

injury or limb amputation. Hence, the question was asked whether this urodele amphibian could develop scars under any circumstance. To verify this, axolotls were injected subcutaneously with either a solution of saline or a dilution of the drug bleomycin every other day during 30 days. These treatments were administered in intact and wounded skin (Figs. 6 and 7). When the treatment was completed, Masson's trichrome and Sirius red colorations were used to check for the presence of fibrosis (Figs. 6 and 7). Masson's trichrome stains collagen (the principal component of fibrotic tissue) in blue while Sirius red enhances the birefringence of collagen fibers and allows the distinction of larger (in red) or smaller (green and yellow) fibers under polarized light (Király et al., '97). In control unwounded animals, repeated injections of saline did not stimulate the production of fibrotic tissue (Fig. 6A–C). Skin integrity was maintained in every control animal and muscles did not seem to be affected by repeated injections of saline. Interestingly, repeated injections of bleomycin diluted at 100 μ g/mL or 1 mg/mL induced the production of fibrotic tissue under the epidermis of intact skin in axolotls just as described by Yamamoto et al. in mouse skin (Yamamoto et al., '99). When diluted at 100 μ g/mL, bleomycin caused tissue fibrosis between the skin basement membrane and the muscles (Fig. 6D). This fibrotic-like tissue was stained pink by Masson's trichrome, suggesting that it was not composed of collagen (Fig. 6D). Under white light, Sirius red also stained the fibrotic tissue in pink (Fig. 6E). However under polarized light, almost no collagen staining was detected in this fibrotic-like tissue (Fig. 6F). As

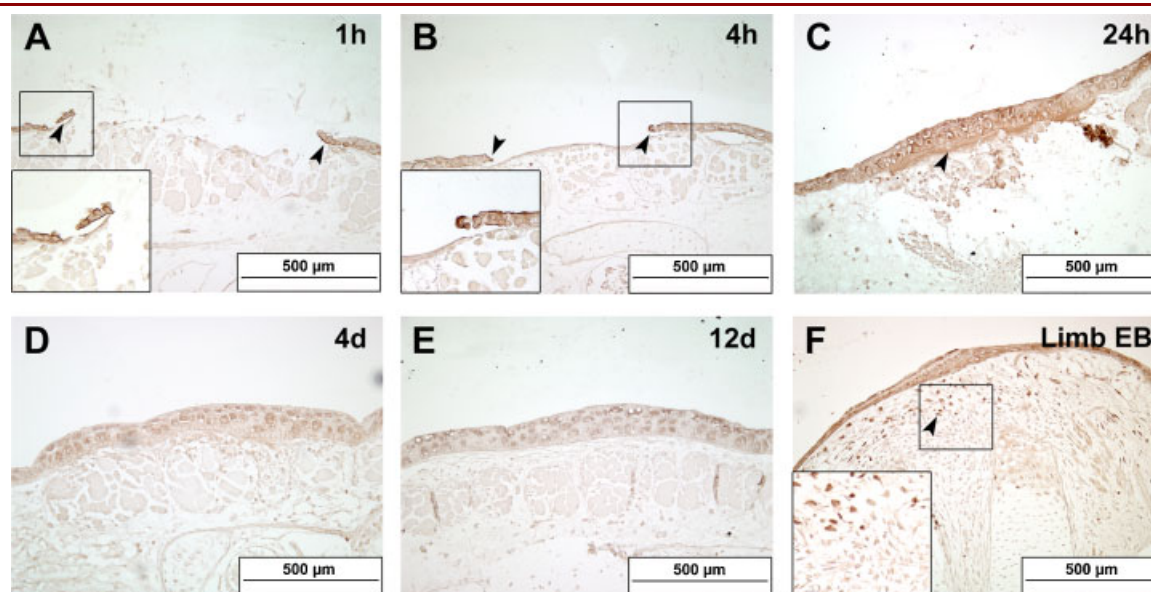


Figure 5. Transforming growth factor β -1 protein expression during wound healing in the axolotl. Immuno-histochemistry experiment showing the presence of TGF- β 1 protein production during wound healing. The presence of TGF- β 1 can be seen, 1 h after wounding, around epidermal cells on the wound's edge (A, arrowhead and inset). TGF- β 1 is detected around cells at the tip of the migrating epidermis 4 h after wounding (B, arrowhead and inset). A strong diffuse signal corresponding to TGF- β 1 is detected in the extracellular matrix underlying the wound epidermis 24 h after wounding (C, arrowhead). Only a weak background signal could be detected 4 days and 12 days after wounding (D, and E). As a control, protein expression was assessed during limb regeneration in axolotl. Many cells in the blastema are positive for TGF- β 1 protein expression corresponding to what was previously published by our group (F and (Levesque et al., 2007)). [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

expected, bleomycin injections at 1 mg/mL induced the production of a greater amount of fibrotic-like tissue than injections made at 100 μ g/mL. At this concentration, bleomycin induced a thicker subcutaneous accumulation of fibrotic-like tissue (Fig. 6G). Additionally, in some animals, bleomycin induced interstitial accumulation of collagen fibers between the muscles. Collagen was present between muscles in control animals as well (blue staining, Fig. 6A) but was found in greater amounts in muscles of bleomycin-injected animals, under the injection site (see blue coloring between muscles in Fig. 6D and G). Sirius red staining enabled the detection of collagen fibers in the region corresponding to the blue interstitial fibers stained with Masson's trichrome (Fig. 6F and I). A few collagen fibers could be detected in bleomycin-induced fibrotic tissue when overexposed (data not shown), suggesting that it may contain minute amounts of collagen. Additional experiments were performed in order to identify the other components of the pink subcutaneous bleomycin-induced scar in the axolotl. Immuno-histochemistry for fibronectin and α -SMA were performed and showed that the tissue was partially composed of fibronectin (Supplementary figure). Results for α -SMA protein expression in skin wounds were negative for every condition tested (Supplementary figure).

Bleomycin injections were also administered concomitantly during skin wound healing to assess whether it would result in abnormal wound healing. After 30 days of treatment, saline injections resulted in normally looking skin with gland cells and epidermal cells present in the skin (Fig. 7A and B). The basement membrane was not completely regenerated by this time as shown above for untreated wound healing (Fig. 2). Treatment with 100 μ g/mL of bleomycin caused the accumulation of a fibrous-like tissue just under the wound site (Fig. 7D and E), which was not observed in saline-treated animals. In addition, the regeneration of the basement membrane was more impeded in the presence of 100 μ g/mL of bleomycin. However, the epidermal layer of the skin looked normal with the presence of gland cells and epidermal cells. At the highest concentration of bleomycin injection, 1 mg/mL, skin wound healing was severely affected (Fig 7G–I). The epidermal layer of the skin was much thicker than the saline or the 100 μ g/mL bleomycin-injected wounds. The basement membrane was completely absent indicating that its regeneration was inhibited by a high concentration of bleomycin. Finally, the muscle under the wound site looked atrophied and or replaced by a fibrous-like structure, which contained some minor collagen fibers (Fig. 7H and I) that are indicative of scar-like tissue.

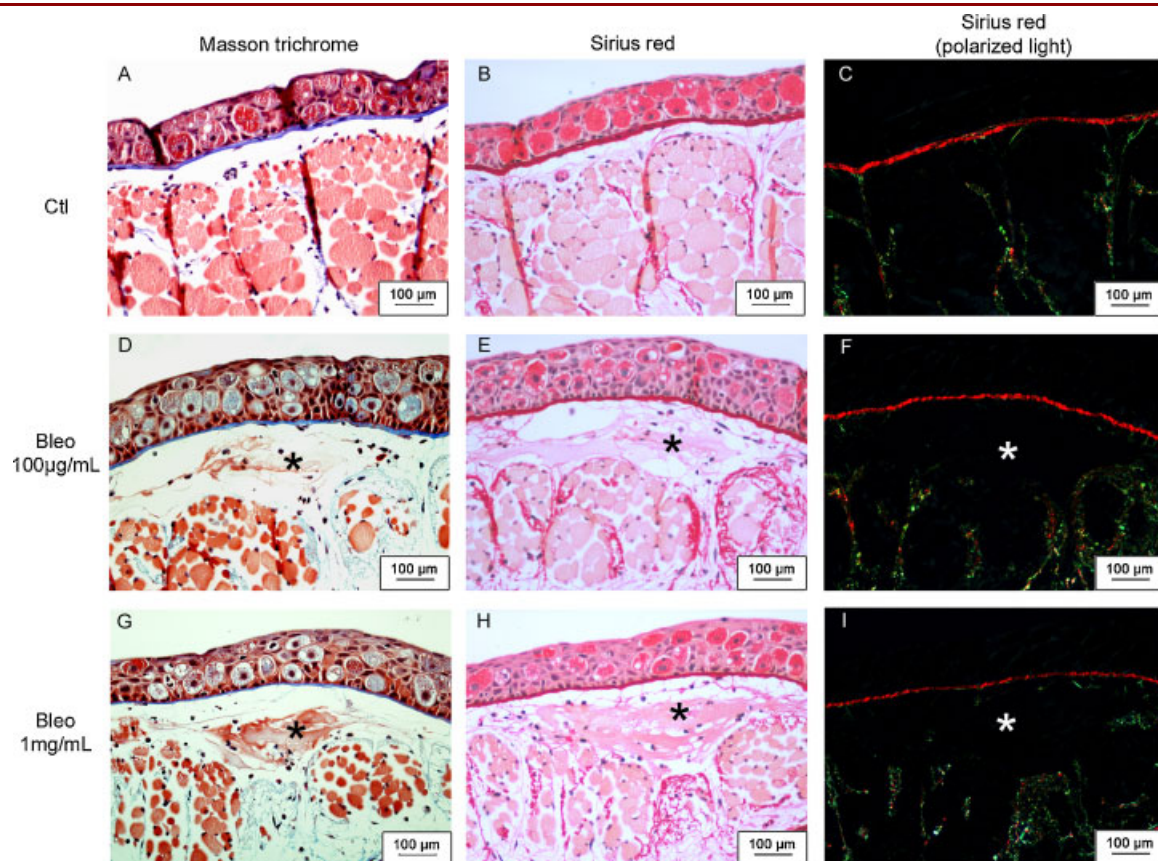


Figure 6. Histological analysis of bleomycin-induced fibrosis in axolotl. Samples were analyzed using either Masson's trichrome (A, D, and G) and Sirius red under white light (B, E, and H) or Sirius red viewed under a microscope equipped with a polarized light (C, F and I). Control PBS injections did not induce tissue fibrosis in axolotl (A, B, and C). Injections of 100 µg/mL bleomycin induced the appearance of light fibrosis under the skin (D, E, and F). Injections of 1 mg/mL bleomycin resulted in the induction of extended fibrosis in axolotls (G, H, and I). Fibrotic tissue is stained pink with Masson's trichrome (D and G) and pink with Sirius red (E and H). Under polarized light, Sirius red stains large collagen fibers red and small collagen fibers green and yellow as seen in the basement membrane of the skin and between muscles (C, F, and I). A few thin collagen fibers could be observed in the fibrotic tissue induced by bleomycin. Black asterisks in D, E, G, and H are positioned on the top of the fibrotic tissue induced by bleomycin. The white asterisks in F and I indicate that the fibrotic tissue indicated with the black asterisks does not contain very much collagen as it appears black under polarized light in Sirius red stained sections. [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

DISCUSSION

Wound healing is a process common to all vertebrates, which takes place to prevent micro-organisms from the outside world to infiltrate the body. In mammalian embryos, this process leads to scar-free healing until the half of the second trimester of gestation (Ferguson and O'Kane, 2004). Later in development and in adults, wound healing is differentially regulated and leads to the formation of a collagenous scar at the wound site (Ferguson and O'Kane, 2004). Wound closure is essential to maintain the integrity of the body but scarring has many drawbacks. Apart from the disruption of appearance, which is of great importance in humans, scars can impair movement and prevent bodily

functions as some structures (i.e. hair bulbs and sweat glands) are not regenerated (Martin, '97).

Unlike mammals, urodele amphibians can heal their wounds without scars and can even regenerate a complete limb after amputation (Wallace, '81; Roy and Lévesque, 2006). The capacity to regenerate and heal wounds perfectly as adults is unique to urodeles among vertebrates. Although frogs are known to regenerate and heal their wounds as larvae/froglets (Suzuki et al., 2005), it is also well documented that they lose the capacity to regenerate their limbs once they metamorphose (Dent, '62). However, there are very few studies that have been published on the ability of adult anurans to heal their wounds. There is,

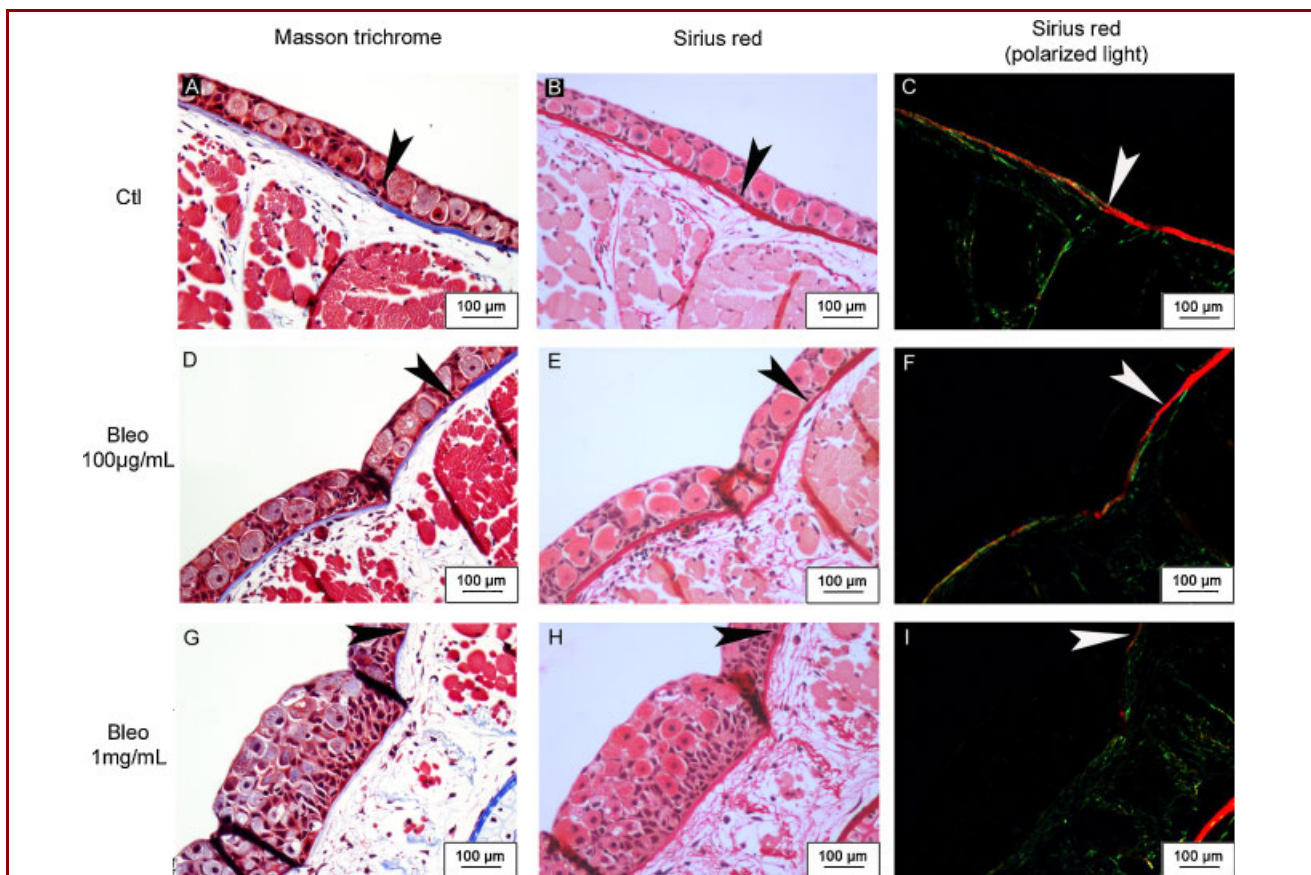


Figure 7. Histological analysis of bleomycin treatment on wound healing. Samples were analyzed using either Masson's trichrome (A, D, and G) and Sirius red under white light (B, E, and H) or Sirius red viewed under a microscope equipped with a polarized light (C, F, and I). Control PBS injections did not induce tissue fibrosis or affect wound healing in axolotl (A, B, and C). Injections of 100 µg/mL bleomycin slowed down the regeneration of the basement membrane and seemed to induce a slight increase in the number of collagen fibers under the skin (D, E, and F). Injections of 1 mg/mL bleomycin resulted in a significant inhibition of the regeneration of basement membrane and a marked increase in the number of collagen fibers under the healed skin (G, H, and I). It also resulted in a thicker epidermal layer, which was about double the thickness (G and H) as the epidermal layers in the PBS (A and B) and 100 µg/mL bleomycin (D and E)-injected wounds. In addition, the muscles displayed more atrophy with the 1 mg/mL bleomycin injections (G and H). The black and white arrowheads indicate the right edge of the wound. [Color figures can be viewed in the online issue, which is available at wileyonlinelibrary.com]

however, one study that did look at the ability of metamorphosed frogs to heal their skin wounds. This study demonstrated that, as mammals, frogs will form a scar after a full thickness excisional skin wound (Baitsell, '16). The ability of axolotls to *perfectly* heal their wounds was often looked at or mentioned in studies focusing on the limb regeneration process but was rarely addressed elsewhere on their body before now. This paper is one of the first to include observations concerning timing of events, cellular movement, and protein expression after excisional wounding in vivo in the axolotl. Tanner et al. recently published a paper in which they described the movement of different skin cells in axolotl wounds in vitro using image correlation spectroscopy (Tanner et al., 2009). They found similar results as ours regarding the time required for wound closure and

epidermal cell migration, mostly keratinocytes, from the edge of the wound (Tanner et al., 2009). Their in vitro approach allowed them to look deeper into the epidermal layers of the skin in live cells, which was not possible in vivo, and permitted them to measure cell movement with precision. However, they were only able to maintain the explants alive for about 72 h, which limited their study to early time points. Our study is focused more on the histological integrity of full thickness skin and how it is reconstituted/regenerated after excisional wounding after different lengths of time. We completed our study at 90 days after wounding as we could no longer detect any differences between intact and healed skin. After wounding one side of the tail with a 1.5-mm biopsy punch, we observed that skin closure took under 8 h in the axolotl. This time-frame corresponds to what was

observed by Satoh et al. and Tanner et al. with a size-matched wound, also in the axolotl (Satoh et al., 2008; Tanner et al., 2009). Wound closure was not accompanied with excessive bleeding or scab formation and it was characterized by rapid migration of the epidermal layer over the wound area. A previous study of limb regeneration by Repesh and Oberpriller has shown that epidermal cells migrate over a fibrin substrate in the hours after amputation (Repesh and Oberpriller, '78). This could also be the case after excisional wounding but this was not investigated in this study.

Axolotls healed their wounds at a faster rate than mammals in which the process of re-epithelialization can last for up to 10 days after wounding (Gurtner et al., 2008). In contrast with another study on axolotl, no accumulation or production of collagen was observed under the skin of the animals at any point during wound healing (Endo et al., 2004). This may result from the technique used to wound the animal or the staining protocols. We used the Masson's Trichrome and Sirius red, which are specific for collagen, and they used Mallory's triple stain, which will stain connective tissue blue as well as collagen. Unlike the results obtained by Endo et al., we did not see any staining for collagen under the wounded area between 5 and 7 days after wounding (Endo et al., 2004). Instead, our histological analysis shows that the collagenous basement membrane of the axolotl skin is starting to be re-established around day 18 and is perfectly re-established around 90 days after wounding. Using this observation, a parallel can be traced between skin wound healing and limb regeneration in axolotls. As in wound healing, the apical epithelial cap (AEC) at the tip of the regenerating limb in urodeles lacks a basement membrane (Christensen and Tassava, 2000). It is now accepted that exchanges of signals between the AEC and the blastema are essential to the process of limb regeneration (Campbell and Crews, 2008). Further investigations would be needed to verify if this is also the case during skin wound healing in urodeles. The basement membrane, which is considered a hallmark of the dermal layer, is not regenerated as early as the epidermal layer of the skin (Fig. 2). The dermis, which contains fewer cells than the epidermis, is mostly populated of fibroblasts, which we started to see near and/or under the edge of the wounds 24–48 h after wounding. The exact origin of these fibroblasts is difficult to determine exactly as they could have migrated up from the connective tissue between muscle cells, which also contains fibroblasts, or from the dermis on the edge of the wound. Our data (Fig. 2E and F) suggest that it is likely from the edge of the wound as we see more fibroblasts near the edges of the wounds at 24 and 48 h after wounding (see black arrowhead in Fig. 2E and F). This is consistent with the work of Endo et al. which reported the migration of dermal fibroblasts 72 h after wounding (Endo et al., 2004). However, one has to be careful when identifying fibroblasts as there are no specific markers that can be used to clearly identify or even label them. We, like others, refer to the cells observed in the dermis as fibroblast based on their flat-like appearance and the fact that they are in the dermis.

Results also show that the neutrophils are not recruited to the site of a skin wound in axolotls. In mammals, these cells are part of the first wave of immune response and are partly responsible for the inflammatory response after wounding (Martin, '97). It has been argued for a long time that a weak immune response correlates with tissue regeneration (Mescher and Neff, 2006). In human embryos, wound healing switches from a regenerative response to scarring when the immune system is being developed during the second trimester of pregnancy (Adzick and Lorenz, '94). The same tendency is observed in anurans, which lose their ability to regenerate their limbs at the beginning of adulthood, when their immune system is completely developed (Mescher and Neff, 2006). However, the exact role of immune cells during the regeneration process is still not elucidated. In the newt, during limb regeneration, there is a general decline of leukocytes (e.g. lymphocytes, neutrophils, eosinophils, basophils, and monocytes) in peripheral blood (Sicard, '83). In this paper, only a few neutrophils were detected 48 h after wounding in some of the axolotls with wounded skin. This is indicative of a very weak immune response after wounding. The presence of neutrophils during the limb regeneration, 48 h after amputation was also assessed. The results obtained are also in agreement with the observations made by Sicard although no histological data were included in his paper: no neutrophils were detected at the tip of the regenerating limb in the axolotl (Sicard, '83). Our data are also in agreement with the work of Ziegels, which looked at melanocytes during skin wound healing in axolotls, where he indicates that he did not observe the presence of macrophages in the first 48 hr after wounding (Ziegels, '71). However, our results following a stab wound indicates that the axolotls are not completely devoid of an immune response involving the recruitment of neutrophils. The difference can be explained in part by the fact that excisional wounds and limb amputations are quite different than a stab wound. When a stab wound is inflicted deep into the muscles, the damaged cells are left in place unlike excisional wounds or limb amputation. Many of these cells will die of necrosis, which is well known to induce inflammation. Hence, the recruitment of neutrophils to rid the wound of cellular debris and potentially from the bacteria that were pushed deep into the tissue as oppose to the situations observed in our excisional wound model or after limb amputation where there is no need to recruit neutrophils to eliminate cellular debris. The immune regulation of tissue regeneration has been overlooked in the past and will be important to consider in the future. In comparison with adult mammals, it seems that axolotls display a diminished immune response characterized by very few neutrophils at the wound site after excisional wounding (Engelhardt et al., '98). In mammals, immune cells are responsible of secreting many cytokines that promote inflammation and tissue fibrosis (Wynn, 2008). The absence of immune cells at the wound site, in our case neutrophils and in Ziegels case macrophages (Ziegels, '71), may be related to the absence of scar formation in urodeles.

However, we cannot say whether the absence of immune cells is directly or partly responsible for the absence of scarring in axolotls.

In mammals, scar formation is mainly mediated by myofibroblasts, specialized cells that express α -SMA, which can contract the wound area and secrete collagen (Hinz, 2007; Wynn, 2008). Our results led us to find that there are very low levels if any of α -SMA-expressing cells in the wound area in axolotl skin. Again, this may be a reason why the axolotl doesn't form any scar when wound healing is completed. TGF- β 1 is able to induce the production of α -SMA during wound healing in mammals (Desmouliere et al., '93). In axolotl wounds, TGF- β 1 was transiently expressed in cells at the forefront of the epidermal layer migrating over the wound area. It was also expressed under the newly formed epidermis in the wound bed 1 day after wounding but was downregulated to undetectable levels 4 days after wounding. This expression pattern is shorter than what was reported in mammals where TGF- β 1 is expressed for several days in the wound area (Kane et al., '91; Levine et al., '93; Frank et al., '96). TGF- β 1 is considered as one of the main regulators of wound healing in mammals (Border and Noble, '94; Martin, '97; Wynn, 2008) and is therefore not a surprise to see it being expressed in other vertebrates such as urodeles. In fact our group has previously shown that TGF- β 1 is expressed during limb regeneration in the axolotl (Levesque et al., 2007). We also demonstrated that TGF- β signaling is essential for limb regeneration (Levesque et al., 2007). TGF- β 1 is also expressed transiently during wound healing in mammalian embryos, which do not form scars (Martin et al., '93). The context of regulation of TGF- β signaling and the presence or absence of unknown co-factors interacting with TGF- β s should be thoroughly investigated in the axolotl. Deciphering this pathway in the axolotl could help explain why TGF- β 1 seems to promote tissue fibrosis in adult mammals and tissue regeneration in mammalian embryos and in salamanders.

Finally, subcutaneous injections of bleomycin induced tissue fibrosis in axolotls. This is interesting, as these animals do not form any scars during normal wound healing. Again, this brings us to establish the comparisons between wound healing and tissue regeneration in mammals and urodeles. A strong argument to distinguish between wound healing and tissue regeneration was previously presented using the example of non-union fracture healing in the axolotl (Roy and Lévesque, 2006; Hutchison et al., 2007). It was shown that, as mammals, axolotls are not able to regenerate a bone gap beyond a certain critical size (Roy and Lévesque, 2006; Hutchison et al., 2007). The conclusion was that regeneration is not an improved form of healing (i.e. superhealing) but a completely different process. The bleomycin experiments demonstrate that axolotls are not resistant to chemically induced fibrosis even if they never form scar tissue during normal wound healing. However, these scar-like structures induced by bleomycin do not contain very much collagen, which is normally abundant in mammalian scar tissue. This shows that axolotls possess all the

molecular pathways to produce tissue fibrosis but suggests that these pathways are not activated after wounding or limb amputation. Interestingly, even the treatment of axolotl skin wounds with bleomycin did not lead to excessive collagen production. We observed a slight increase in collagen staining with Sirius red under polarized light similar to what was observed when treating unwounded skin with bleomycin. However, bleomycin treatment did slowdown considerably the regeneration of the basement membrane during wound healing and resulted in thicker looking skin. Future experiments will be needed to sort out the differences and similarities between the genetic programs regulating wound healing and regeneration in salamanders and those regulating mammalian wound healing.

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