Evolutionary conservation of leptin effects on wound healing in vertebrates: Implications for veterinary medicine

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Abstract

In mammals, the cytokine hormone leptin promotes wound healing by increasing inflammation, cellular recruitment, angiogenic regrowth and re-epithelialization; however it is not known whether leptin has conserved actions on wound healing in other vertebrates. Here, we tested the hypothesis that leptin promotes both the quality and speed of wound healing in the South African clawed frog, *Xenopus laevis*. First, fluorescent immunohistochemistry using a polyclonal antibody specific to *Xenopus* leptin showed that in juvenile dorsal skin, leptin protein is expressed in the dorsal epidermal layer, as well in blood vessel epithelial cells and sensory nerves that run along the base of the dermis. Injection of recombinant *Xenopus* leptin (rXleptin) stimulates phosphorylated STAT3, indicative of leptin-activated JAK/STAT signaling in the epidermis. Similar to mammals, leptin protein expression increases at the wound site after injury of the epidermis. We then cultured 'punch within a punch' full thickness dorsal skin explants in three doses of rXleptin (0, 10 and 100 ng/mL), and showed that leptin treatment doubled the rate of wound closure after 48 hr relative to skin punches cultured without leptin. Food-restriction prior to wound explant culture reduced the amount of wound closure, but leptin injection prior to euthanasia rescued closure to similar to control levels. Leptin treatment also signficantly reduced bacterial infection of these epidermal punches by 48 hr in culture. This study shows that leptin is likely an endogenous promoter of wound healing in amphibians. Leptin-based therapies have the potential to expedite healing and reduce the incidence of secondary infections without toxicity issues, the threat of antibiotic resistance, or environmental antibiotic contamination. The conservation of leptin's actions on wound healing also suggest that it may have similar veterinary applications for other exotic species.

1 Introduction

As the number of important amphibian populations grows in breeding and conservation collections in zoos, conservation programs, and the pet industry, there is a recognized need for better therapies for the treatment of injuries and infections (1–3). In amphibians, the skin is an important organ for respiration and immunity, but it is also easily injured and vulnerable to infection. Current treatments for skin wounds or diseases in captive amphibians are limited to antibiotics and antifungals, though pharmacokinetics and effective dosages are not well understood (4–6). While these are effective at preventing infection, they do not explicitly accelerate wound healing. Overuse of antibiotics also

2 Methods

2.1 Experimental Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Washington State University. *Xenopus laevis* were obtained from Xenopus1 (Dexter, MI) as larvae and raised to 6-8 months post metamorphosis. Animals of this age have recovered much of their immune function after the downregulation that occurs during metamorphosis but still retain full ability to regenerate skin scar-free after injury (9,10). Frogs were fed every other day unless noted otherwise. In studies of *in vivo* wound closure in *Xenopus* juveniles, both incisional and square wounds form a simple, undifferentiated wound epidermis within 24-48 hrs of the wound occurring (9,28). This is followed by epidermal thickening with mononuclear cells and cells from subdermal layers infiltrating to remodel the dermis (9,28,29). Scarless wound healing takes approximately 2 months in the juvenile, but remodeling takes much longer in the adult frog and will scar (9).

2.2 Leptin and leptin receptor expression in *Xenopus* dorsal skin

To determine the expression profile of leptin protein in uninjured dorsal skin, we dissected skin from juveniles and fixed it in 3% PFA/1% glutaraldehyde overnight at 4°C; approximately 1 mm x 1 mm squares were cut from dorsal tissue and sectioned for immunohistochemistry (n=6). To determine where leptin receptor is expressed in the skin and to see if leptin activation of the receptor activates JAK/STAT signaling in the skin, 6-8 mo juvenile *X. laevis* were injected with either sterile saline or 200 ng/g body weight recombinant *Xenopus* leptin protein (rXleptin) intraperitoneal (IP). Recombinant *Xenopus* leptin was produced following methods in Crespi and Denver (22) and purified using the Ni-NTA Purification System (K95001, Invitrogen, Waltham, MA) in hybrid conditions following manufacturer's instructions. After 6 hr, frogs were euthanized and the dorsal skin was fixed and prepared for immunohistochemistry as described above (n=3/injection type).

2.3 *In vitro* wound healing assay culture conditions

To determine the effect of upregulation of leptin signaling on cutaneous wound healing, we developed an *in vitro* culture assay in which we incubated skin explants with or without recombinant *Xenopus* leptin. Six- to eight-month post-metamorphic *X. laevis* frogs were euthanized via benzocaine overdose followed by double pithing. Following methods adapted from Meier et al. (30), donut-shaped "punch-in-a-punch" *in vitro* wounds were created with cut tissue on the outside and inside edges. Briefly, dorsal skin was dissected from the neck and down the lateral lines to the lower back, then rinsed three times with sterile phosphate-buffered saline (PBS). The dorsal skin sheet was then placed on a sterile cutting mat, with care taken to minimize mucus production and damage to the hypodermis. After allowing the skin to dry briefly and excess water to evaporate, a 4 mm tissue biopsy punch (World Precision Instruments, Sarasota, FL USA) was used to remove circular sections of skin which then had 1 mm biopsy punches removed from the center to create a wound and forming the skin donut. With care taken not to stretch the wound in the center, these tissues were placed in 24-well plates with either Matrigel (Corning Life Sciences, Glendale, AZ USA) or ECM Gel (a Matrigel equivalent; E1270, Sigma-Aldrich, St. Louis), both of which are produced from Englebreth-Holm-Swarm murine sarcoma; 250 µL of each were diluted to 2.5 mg/mL protein with 1x L-15 media. After allowing the skin donut explants to adhere to the basement layer for approximately 3 minutes, each well was flooded with 1 mL sterile L-15 with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin. Tissue was incubated at 28°C. For immunohistochemistry, tissues were fixed at 0, 24, 48 hours post injury (hpi) with 3% PFA/1% glutaraldehyde overnight at 4°C.

injections/animal; n=8/fed status/injection type). All samples for this experiment were processed within a few days. The day following the last injection, animals were euthanized by benzocaine overdose and the dorsal skin was removed for wound healing assays as described above. Dorsal skin donuts were incubated with or without rXleptin (100 ng/mL leptin) in ECM Gel in L-15 media for 48 hpi, imaged at 0, 24, and 48 hpi, and fixed in 3% PFA/1% glutaraldehyde overnight at 4°C for immunohistochemistry. Only animals that had explants in all treatments and did not have visible microbial infection were included in analysis (n=8/treatment group. We also saw variation in initial area of wounds similar to the dose-response experiment with no significant difference between treatments (0.302-0.722 mm^2, one-way standard least squares ANOVA, $F_{3,50}$=1.7113, p=0.1766). In this experiment we compared the slope of wound sizes from 0 to 48 hpi for each individual; measurements made were not blind to treatment groups. We then used three-way standard least-squares ANOVA to determine effects of nutritional status, pre-treatment, culture condition, and their interactions on wound closure; if 2- and 3-way interactions were not significant, they we removed them from the model. Significant differences among treatment groups within each time period were then evaluated using Tukey HSD multiple comparisons tests ($\alpha = 0.05$).

2.7 Effect of leptin on microbial growth in skin explant culture

During the wound healing experiments, we observed that explants cultured without leptin became microbial infected more frequently compared to those cultured with leptin (note that explants that were infected were not included in the above analyses). As a post-hoc analysis, we compared infection frequency (i.e., whether culture media was visually cloudy) at 48 hr of culturing between saline and rXleptin using a likelihood ratio test. Because L-15 media changes color with pH we could not use absorbance assays to quantify the extent of infection. We included all explants cultured across experiments except for food-restricted animals because there was only 1 infected well in this set of explants. Sample sizes per dose were: 0 ng/mL ln=70, 10 ng/mL n=38, 100 ng/mLn=70.

2.8 *Xenopus* leptin antibody and pre-absorption

To detect leptin protein, we produced a *Xenopus*-specific leptin polyclonal antibody raised in rabbits injected with purified recombinant *Xenopus* leptin protein in the Washington State University Center for Reproductive Biology Core Facility. To ensure that this anti-leptin polyclonal antibody (anti-Leptin) was binding to leptin specifically, the anti-Xleptin was incubated 1:10 with 400ng/μL rXleptin protein at room temperature for 1 hr prior to primary staining as described below (pre-absorption).

2.9 Immunohistochemistry

We used immunohistochemistry to describe the cellular localization of leptin protein and phosphorylated STAT3 (pSTAT3) as an indicator of leptin receptor activation within the skin (injured and not injured), and to detect proliferating cells. All fixed tissues were dehydrated with methanol for storage at -20°C and then rehydrated stepwise with PBS with 0.01% Triton X-100 (PBST). They were bleached in a solution consisting of 5% 30%-hydrogen peroxide and 5% formamide in PBS on a light table for 2 hr until granular glands were pale cream in color and all other tissue was white. Tissues were rinsed 3 x 2 min in PBST followed by 2 x 30 min permeabilization in PBST. Tissues were blocked in 10% blocking reagent (Roche 11096176001, Sigma-Aldrich, St. Louis, MO) in PBST for 1 hr at room temperature, then incubated overnight at 4°C with primary antibody in 10% blocking reagent in PBST at the following concentrations: 1:2000 anti-Xleptin, 1:250 anti-phosphohistone H3 (PH3, ab14955, Abcam, Cambridge, UK), 1:500 phospo-

All explants partially closed over time (time, $F_{1,25} = 13.4764$, $p = 0.0011$), but those cultured with 10 and 100 ng/mL leptin closed to a greater extent at 24 hpi; only 100 ng/mL closed significantly more than the control at 48 hpi (Figure 5; ANOVA Treatment $F_{2,25} = 7.2136$, $p = 0.0034$).

3.4 Effects of nutritional state and leptin administration on skin explant wound closure

On average, extended food-restriction resulted in 18% weight loss (wet weight mean ± S.D., start: 11.20 ± 3.61 g, end: 9.20 ± 2.69 g), while the fed daily group maintained their body weight (8.61 ± 3.66g, end: 8.67 ± 3.32g). The effect of food restriction on wound closure depended on leptin pre-treatment (ANOVA fed status*pre-treatment, $F_{4,41} = 5.3898$, $p = 0.0253$). In explants from frogs fed daily, there was no effect of leptin pre-treatment or leptin in the culture media on wound closure rate, although explants treated with leptin in culture exhibited the fastest closure rates by 48 hpi (Figure 6A). By contrast, in explants from food-restricted frogs, leptin pre-treatment significantly increased wound closure rates compared to those injected with saline (Figure 6B). Although leptin addition to the culture media tended to further increase wound closure rates, we did not detect an overall effect of *in vitro* leptin treatment (Figure 6B).

3.5 Effect of leptin on microbial growth of skin explant culture

Across both experiments analyzed in this post-hoc analysis, leptin treatment in the culture media decreased the number of culture wells with microbial growth (likelihood ratio test, p=0.0202; note that infected wells were excluded from the above analyses). Leptin at 100 ng/mL reduced the proportion of wells infected by almost half when compared to wells with no exogenous leptin added (Figure 7).

4 Discussion

In this study we show that leptin protein is expressed in the skin, released after injury at the site of a wound, and improves wound closure in *X. laevis* juvenile frogs. In uninjured skin, leptin protein was present mainly in the nerves,and endothelial cells of blood vessels in the hypodermis prior to injury, yet leptin activates JAK/STAT signaling (leptin-induced pSTAT3 upregulation) most strongly in the epidermis, but also dermis, hypodermis, and the apical surface of the granular glands. After injury, we localized leptin and pSTAT3 in all three skin layers within minutes after injury with the highest concentration in the epidermis and dermis at the edge of the injury. Lastly, we showed that the rate of wound healing in our *in vitro* assays increases with elevated leptin signaling; and when wound healing is slowed in explants harvested from food-restricted frogs, systemic leptin administration rescues wound closure ability to rates similar to that of fed frogs. These findings complement studies showing upregulation of leptin mRNA at the site of wounds in larvae (24-27), and provide the first functional evidence that leptin signaling modulates wound healing in amphibians.

When combined with findings showing topical leptin enhances wound healing in mammals and lizards (14,16,34), our findings are consistent with the idea that leptin signaling is an ancient, evolutionarily conserved modulator of wound healing in vertebrates with both endocrine and paracrine signaling. In both *ob/ob* and food-restricted mice, wound closure is delayed compared to wild-type mice but topically applied leptin increases STAT3 phosphorylation and reverses this outcome (16,35). In our study, we show that leptin is present in neurons and endothelial cells of the blood vessels in the skin, which allow immediate leptin release at the site of the wound, although we cannot rule out delivery of leptin through blood to the site of the wound, We also observed that food restriction slowed wound healing relative to well-fed animals, as shown in mammals (36). The lack of effect of leptin in fed-daily frogs could be due to elevated endogenous nutritional modulators or

localization of leptin protein-ir and the neural marker AαT-ir shows that leptin is in nerves throughout the skin, some of which connect to granular glands. This suggests that neurosecretory leptin could have a role in mediating antimicrobial peptide (AMP) production or secretion. AMPs are one of the most important immune defenses against skin infection, and given that microbial growth was reduced in frequency in leptin-treated explants, it is possible that there was an increased release of AMPs in our leptin-treated explants. Leptin has been shown to mediate secretion, particularly mucous production/secretion, in other mammalian contexts (44,45), so it is possible that leptin enhanced wound closure in our experiments through these cytoprotective and anti-microbial actions.

We also showed leptin-ir cells in blood vessels and in cells that have not been described in the hypodermis layer. The hypodermis of mammalian skin contains a layer of subcutaneous fat that secrete factors, such as leptin, to modulate the function of dermis and epidermis which is the major source of leptin in mammals, but amphibians lack an organized subcutaneous fat layer (46). While we did not conduct histology in skin from fed daily or food deprived frogs, we plan on repeating that study to determine if these leptin-positive cells are indeed scattered fat cells, which could be another source of leptin that varies with nutritive condition in frogs. This study highlights potential modulatory roles of hypodermis in wound healing in frogs, a layer that is largely ignored in the literature (46).

Because we focused on wounded skin explants in this study as a first test of leptin actions during wound healing, there were some limitations of our ability to fully explore the roles of leptin signaling. For example, it is possible that leptin treatment alone was not enough to stimulate mitosis in our culture conditions, or that leptin acts as a mitogen during later stages of wound healing, after the 48hpi period we observed. Leptin depletion in the tissue by 48 hpi *in vitro* limited the duration of this study to explore actions that extend in later stages of wound healing. In mammals, leptin mRNA has been shown to be upregulated through several days after a wound (15) when it continues to promote keratinocyte proliferation and angiogenesis (14,16,35). Indeed, leptin27). Leptin protein expression in endothelial cells of blood vessels in the hypodermis as we report here, suggests leptin plays a role in angiogenic regrowth into a wound. Furthermore, endocrine sources of leptin could sustain leptin-mediated processes involved with these later stages of wound healing. This study confirms that leptin is present and is likely a paracrine, neurosecretory, and endocrine signal that promotes wound closure in amphibians, justifying further work is needed to elucidate through which mechanisms it is acting and to confirm these effects *in vivo*.

These findings have important implications for veterinary care of captive amphibians. Hundreds of amphibian species of are kept as pets, in zoos, including assurance colonies of endangered or threatened species of amphibians (47), and in the research laboratory (e.g., *Xenopus*). In transport or captivity, amphibians are vulnerable to injuries, and these wounds are often fatal due to secondary infections from ubiquitous gram-negative bacteria, such as *Aeromonas*, combined with sickness-induced anorexia (1–3). Furthermore, fungal pathogens such as *Batrachochytrium dendrobatidis* or *B. salamandrivorans*, invade the skin and prolonged infections are highly lethal in many species of frogs and salamanders (6,48-50). Factors that improve wound healing and immunity broadly across amphibians are needed (3), especially those that do not cause adverse side effects or concerns about persistence in waste waters. This study provides evidence that supports leptin as an emerging candidate for novel prophylactic or post-injury therapies that veterinarians, zoo curators, and care givers can use to accelerate wound healing to improve the health and survival of amphibians. The fact that IP injection of leptin, which can be done quickly with minimal handling and discomfort, increases wound healing as much or more than topical application widens the application of this to fully aquatic species, especially aquatic species where topical applications could be diluted. Because

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11 Figure Legends

Figure 1. Leptin is expressed in nerves and blood vessels in the dorsal skin of *X. laevis* juveniles. **(A)** Leptin protein is expressed in blood vessels (BV) and nerves (N) in the in the dermis and hypodermis. Only background fluorescence from granular glands (G) is present in tissue stained with antibody that was incubated with recombinant *Xenopus* leptin protein (4 µg) prior to staining (pre-absorbed). Scale bars 100 µm. **(B)** Leptin is expressed in the endothelial cells of a large blood vessel (BV) in the hypodermis and in unknown scattered cell bodies (solid arrows). Leptin also co-localizes with acetylated alpha-tubulin (AαT, yellow), a neural marker in axons and some nerve cell bodies (hollow arrows). Granular glands are autofluorescent after bleaching and clearing at 488 nm (as seen in the AαT panel). Scale bars 100 µm. **(C)** Leptin protein co-localizes with axons innervating a granular gland. **(D)** Cross section showing leptin in the endothelial cells of a blood vessel in the hypodermis, up against the stratum corneum (SC) of the dermis. Scale bar 25 µm.

Figure 2. Leptin stimulates JAK/STAT signaling in skin. Leptin-induced phosphorylated STAT3-ir (pSTAT3-ir, yellow) in 16 µm sections of juvenile *X. laevis* dorsal skin; skin was fixed 6 hr after intraperitoneal injection with **(A)** saline or **(B)** leptin. Leptin stimulates pSTAT3-ir strongly in the epidermis, and at the apical end of the granular glands and hypodermis (representative image from n=3). Scale bar 100 µm. n=3). Epidermis (E), dermis (D), stratum corneum of the dermis (SC), and thin hypodermis (H). Scale bar 50 µm.

Figure 3. Leptin protein is rapidly localized to injured skin. (A) Whole mount of leptin protein (aqua) and acetylated α-tubulin (AαT, yellow) at the time of injury in juvenile *X. laevis* dorsal skin. Dotted line indicates injury at the center of the donut-shaped explant. Leptin is highly expressed in blood vessel and nerve tissue (indicated by AαT) associated with the injury site. Granular glands are autofluorescent after bleaching and clearing at 488nm (as seen in the AαT panel). Scale bar 100 µm. **(B)** Leptin protein expression in 16 µm cross sections of juvenile *X. laevis* dorsal skin after injury. Leptin expression (aqua) is concentrated in the injured tissue at 0 and 24 hpi and nearly depleted by 48 hpi *in vitro* except for some localized in the epidermis (DAPI, magenta). Epidermis (E), dermis (D), stratum corneum of the dermis (SC), and thin hypodermis (H). Arrow in 24 hr panel indicates blood vessel. Scale bars 100 µm. **C)** Leptin is most highly expressed in the central dermis after injury, but expression within 300 µm the injury site in all skin layers is depleted by 48 hpi in culture (standard least squares ANOVA, time point, $p < 0.0001$).

Figure 4. pSTAT3-ir increases after full thickness injury of dorsal skin. (A) The highest pSTAT3 activation was associated directly with the injured edge of the explant (integrated density: 589,578 ± 201,764 ADU, n=3). **(B)** By contrast, the end of tissue explant that was non-injured and cut after fixation had less pSTAT3-ir activation (integrated density: 267,042± 45,092 ADU.

Figure 5. Leptin treatment (0, 10, 100 ng/mL) in culture media significantly improved wound healing in juvenile *X. laevis* dorsal skin explants through 48 hours in culture. (A) At 24 hr, both 10 ng and 100 ng/mL treatments significantly increased wound closure compared to the control, and by 48 hr the 100 ng/mL treatment continued to improve wound healing compared to the control (repeated-measures MANOVA, treatment=0.0034, Tukey HSD, $p < 0.05$, n=12/treatment). **(B)** Representative samples of dorsal skin explants in culture, showing the interior wound of the "punch-within-a-punch" donut-shaped biopsy. Wound closure measured as the total area reduction of the central wound compared to 0 hpi. Scale bars 0.5 mm.