DAVID GEFFEN SCHOOL OF MEDICINE UCLA

Investigating the Role of Autophagy Proteins LC3 and ATG7 in Wound Healing

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Purpose

Chronic wounds have a significant impact on a substantial number of Americans today. The etiology of these wounds is often multifactorial, not fully understood, and further complicates the healing process. Patients affected by these chronic wounds are at an elevated risk of infection, limb loss, and suffer from a lesser quality of life. Autophagy (ATG), the recycling of excess cellular material, has a potential role in wound healing. The purpose of this study is to investigate the role of autophagy proteins, specifically, LC3 and ATG7, to better understand the etiology and potentially optimize the healing of these chronic wounds using both mouse and human models.

Background

The normal wound healing process undergoes four primary phases: hemostasis, inflammation, proliferation, and remodeling. However, chronic wounds do not reach the final remodeling phase and remain in one of the prior phases despite undergoing standard treatment. This subsequently results in chronic pain, lower quality of life, and increased risk of infection and amputation.

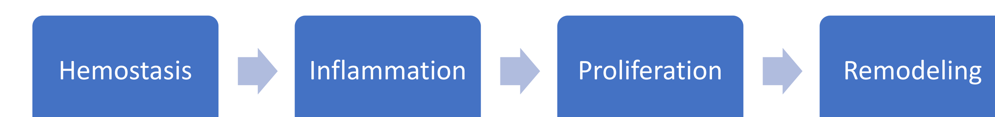

Figure 1: Phases of normal wound healing progression.

Autophagy is a standard catabolic process responsible for degrading and recycling damaged, dysfunctional, and excess cellular material and metabolites allowing cells to prioritize and allocate resources accordingly. This process plays a significant role in essential functions, such as cell metabolism, signaling, differentiation, development, and apoptosis. Collectively, these functions are in turn directly involved in reproduction, maintaining homeostasis, and embryonic development, among others. These autophagic functions also play a role in mounting an immune response, such as cytokine secretion, antigen presentation, T-cell survival, and activation. Impaired autophagy has been linked to cancer, neurodegeneration, diabetes, and increased susceptibility to infection and autoimmune diseases.

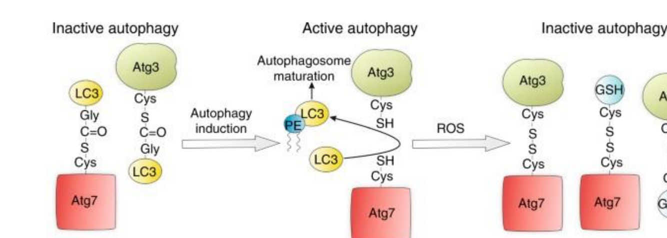

Figure 2: Simplified diagram of autophagy pathway (Frudd et al., 2018).

There are two autophagy proteins in focus in this study, LC3 and Atg7. LC3 (microtubule-associated protein 1A/1B light-chain 3 protein) is responsible for forming the autophagosome around the target cellular component. Atg-7 (autophagy related 7 protein) is responsible for initiating autophagic degradation. Other proteins being studied are NF-KB, CD3, CD4, among others.

Methodology

This study involved both mouse models and human specimens. All studies were approved by UCLA, GLA VA, and OVMC and performed in accordance with Princeton University or UCLA Institutional Animal Care and Use Committee protocols.

Mouse models (Atg7F/F vs. Ubc.Atg7F/F)

The wound healing of ATG7 mice (ATG proficient, control group) and Ubc.ATG7 mice (ATG-deficient, knockout group) were compared. The study utilized both histologic and plasma samples in this study.

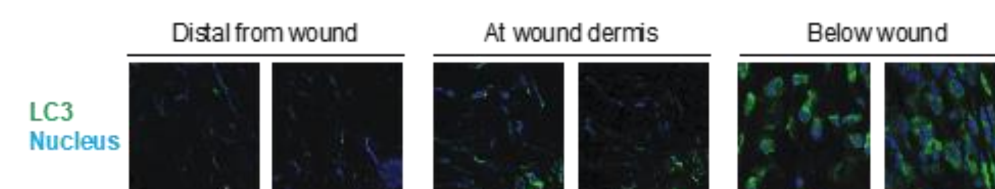

Figure 3: LC3 presence in various locations with respect to a wound using immunofluorescence.

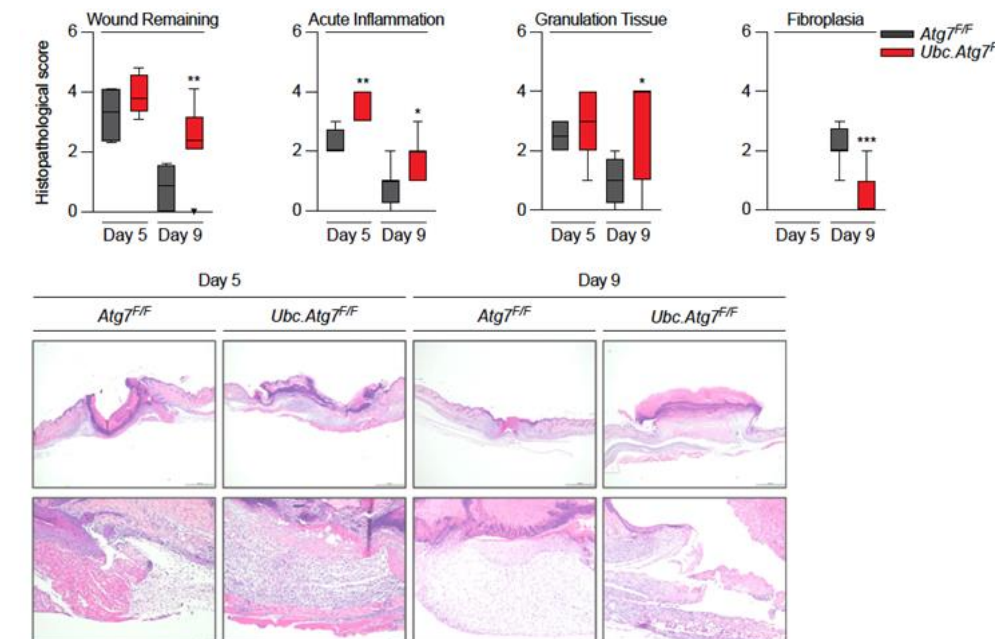

Figure 4 (Top): Quantification of healing progression on days 5 and 9 using histopathological scoring with corresponding histologic slides below **(Figure 5)**.

Samples of intact skin and wounds were collected via punch biopsy 3.5 or 5 mm from clean, hairless skin on the backs of each mouse. Excised tissue subsequently underwent cryosection for preparation to undergo immunohistochemistry (IHC) and immunofluorescence (IF). Protein intensity was quantified using the ImageJ program. After quantification, T-test was performed. Histopathologic scoring was also utilized to semi-quantify tissue samples.

Gel electrophoresis, specifically Western blotting, was used to measure levels of Atg7 and LC3 proteins along with other immunologic proteins using the following primary antibodies: ATg5-ATg12 conjugate, Atg7, CD4, b-actin, a-tubulin, GAPDH, FSP1, CD3, MAGP2, LC3B, MPO, NFkB2 (p100, p-52), IKKb, NF-kB (p65), phospho-NF-kB (Cell Signaling 3033S), IKKa, PCNA.

Gross observation of wounds was also utilized for collecting daily wound measurements. Splint was applied around wound due to mouse skin having a tendency to prematurely contract, which may obscure the accuracy of wound measurements.

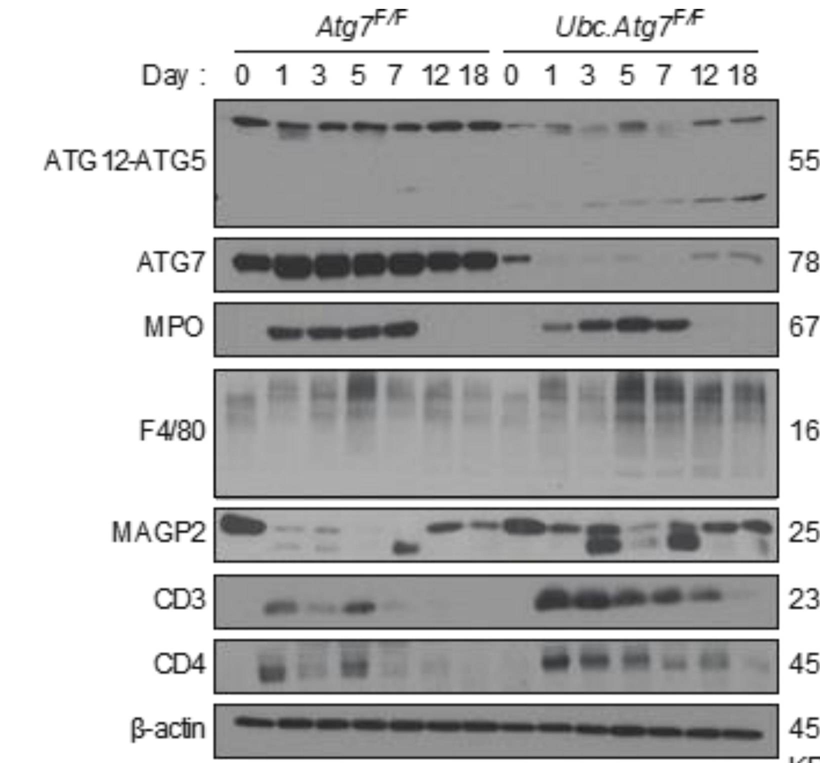

Figure 6: Western blot measuring and comparing levels of various proteins during wound healing progress.

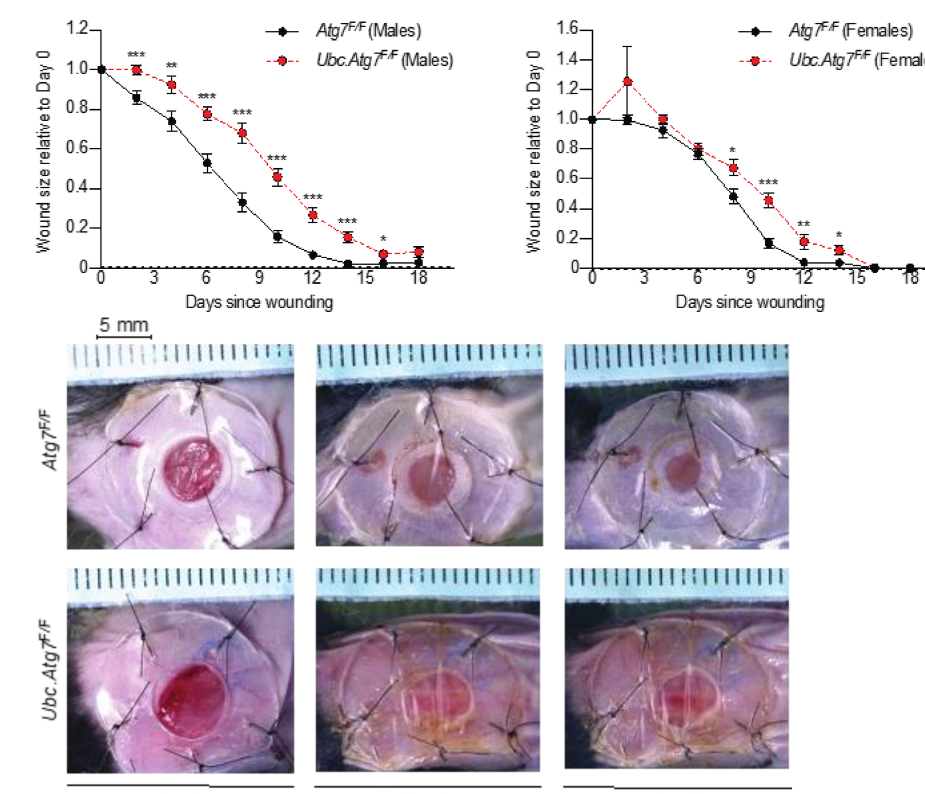

Figure 7 (Top): Comparison of measurements during wound healing progress with corresponding wound imaging below **(Figure 8)**.

Human Specimens (Acute vs. Chronic Wounds)

There was no change in the standard treatment for the volunteers. Samples were harvested from both excisional debridement and amputation specimens. These specimens then underwent similar analytic techniques as the mice specimens. All patients underwent eligibility screening. The 2 primary inclusion criteria are age (>18 years old) and having at least 1 chronic wound (≥4 weeks but <104 weeks). There were also multiple exclusion criteria including but not limited to: wound undergoing living cellular therapy, PDGF, dermal substitute within 30 days of screening along with active Charcot arthropathy and recent malignancy.

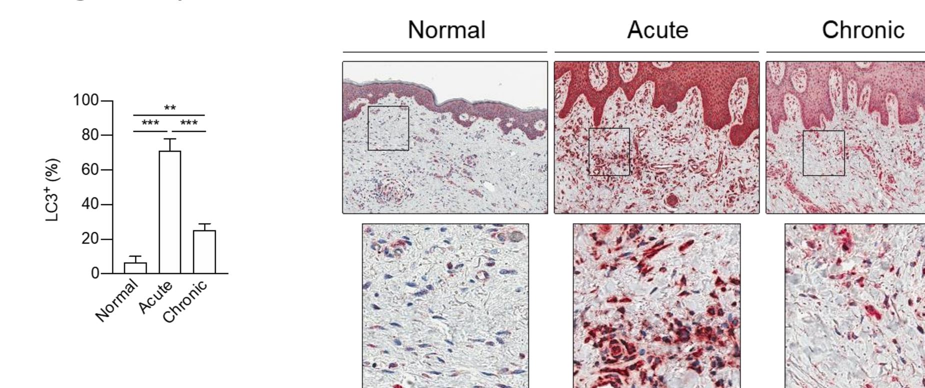

Figure 9 (left): Comparison of LC3 levels in normal skin, acute, and chronic wounds with corresponding image on right **(Figure 8)**.

Results and Discussion

Regarding the mouse models, ATG-deficient mice demonstrated a greater delay in wound healing compared to control group. Pro-inflammatory cytokines were also increased in ATG-deficient mice. LC3 levels were expressed at higher levels around the wound compared to intact skin distal from the wound. In an addition, there was increased levels of NF-KB in autophagy impaired mice. ATG-deficient mice also showed elevated and persistent levels of CD3 and CD4 along with elevated levels of monocytes and macrophage cells.

Chronic wounds from human specimens also demonstrated an elevated level of LC3 proteins. Both LC3 and autophagomes were also elevated in both acute and chronic wounds, highest in acute, compared to normal skin.

Given the ATG-deficient mice and chronic wounds of human specimens having a similar trend of autophagy protein expression, the data suggests autophagy plays a role in wound healing. Of note, autophagy impaired mice showed a significant delay in wound healing along with elevated inflammatory proteins. Though inflammatory proteins are a normal part of the wound healing process, ATG-deficient mice had an extended period of elevated inflammatory proteins compared to control group, which is consistent with chronic wounds unable to progress past the inflammatory phase.

Conclusion

The chronicity of these non-healing wounds is not well understood, and the etiology is often complex and multifactorial. Attaining a better understanding of the cellular and biomolecular processes of wound healing will potentially allow the development of new and more efficient treatments of these chronic wounds.

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Level of Evidence: IV