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Inhibition of IRE1 Results in Decreased Scar Formation

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Abstract

Wound healing is characterized by the production of large amounts of protein necessary to replace lost cellular mass and extracellular matrix. The unfolded protein response (UPR) is an important adaptive cellular response to increased protein synthesis. One of the main components of the UPR is IRE1, an ER transmembrane protein with endonuclease activity that produces the activated form of the transcription factor XBP1. Using luciferase reporter mice for *Xbp1* splicing, we showed that IRE1 was up-regulated during excisional wound healing at the time in wound healing consistent with that of the proliferative phase, when the majority of protein synthesis for cellular proliferation and matrix deposition occurs. Furthermore, using a small molecule inhibitor of IRE1 we demonstrated that inhibition of IRE1 led to decreased scar formation in treated mice. Results were re-capitulated in a hypertrophic scar mouse model. These data help provide a cellular pathway to target in the treatment of hypertrophic scarring and keloid disorders.

Keywords

Hypertrophic scar; scar thickness; unfolded protein response; IRE1; Xbp1

INTRODUCTION

Cutaneous wound healing is a well-studied process consisting of three integrated and overlapping phases. The inflammatory phase is initiated almost immediately post-wounding marked by neutrophil activation, and monocyte and lymphocyte recruitment. This process

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CONFLICT OF INTEREST DISCLOSURE STATEMENT

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peaks at 3–5 days following wounding and persists beyond full re-epithelialization. The proliferative phase begins as early as 3 days post-wounding, peaks at 1–2 weeks and lasts up to a month following injury. This phase is characterized by cell division and new matrix deposition. The final phase of wound remodeling consists of collagen degradation and synthesis which acts to reorganize the collagen matrix in a scar. This process begins soon after re-epithelialization occurs and persists for up to a year or more following wounding¹. Overall, wound healing is a highly anabolic process with copious new protein synthesis required for the production of new extracellular matrix formation and cell proliferation.

The unfolded protein response (UPR) is a signaling pathway that serves primarily to maintain endoplasmic reticulum (ER) homeostasis^{2,3}. During physiologic and pathophysiologic perturbations, the UPR activates a series of adaptive mechanisms to balance the protein folding demands with the protein folding capacity of the ER. The UPR has also been implicated in apoptosis following periods of prolonged and severe ER stress^{4,5}. Therefore, in the development of therapeutic approaches targeting the UPR, candidate molecules targeting this pathway must take into account the therapeutic context in order to achieve the desired clinical goal⁶.

The UPR consists of three major pathways: 1) IRE1, 2) ATF6 and 3) PERK. All three proteins are associated with the ER membrane and are bound to BiP (binding protein, glucose regulated protein 78kDa) in an inactive form under non-ER stress conditions⁷. As described in one model of regulation, the accumulation of misfolded proteins in the ER causes preferential binding to BiP, leading to dissociation and activation of the UPR components. Generally, activation of the UPR leads to activation of the transcriptional program mediating expansion of the ER and increasing the protein folding machinery within the ER. In coordination with these processes are other translational controls that transiently decrease the influx of proteins into the ER^{8,9}.

IRE1 is a highly conserved protein that was initially described in yeast and is ubiquitously expressed¹⁰. Activation of IRE1 results in a nonconventional splicing event in which a 26 nt sequence is spliced from *Xbp1* mRNA resulting in a translational frameshift and production of an active transcription factor (XBP1s) that targets a specific set of genes. Activated IRE1 can also degrade mRNAs that become associated with the IRE1 complex under ER stress. Depending on the context, this process, referred to as regulated IRE1-dependent decay (RIDD), can also regulate apoptosis¹¹.

Since wound healing requires a significant increase in protein synthesis, we hypothesized that the UPR plays an important role during this process. Our previous data showing increased IRE1 activation in keloids, a disorder of excessive wound healing, further suggested a role for IRE1 during normal wound healing¹². We show here that IRE1 was coordinately activated with the proliferative phase of wound healing, when protein synthesis for cellular proliferation and matrix deposition was at its peak. We further show that inhibition of IRE1 with 3,6-DMAD, a highly specific inhibitor of IRE1¹³, decreases scar thickness formation in a “humanized” mouse excisional wound model and in a mouse hypertrophic scar model and does not affect wound strength.

MATERIALS AND METHODS

Animal Care and Procedures

All animal protocols were in accordance with protocols approved by the Stanford University Institutional Animal Care and Use Committee (IACUC). *Xbp1-Luc* transgenic mice were previously described¹⁴. C57Bl/6 mice were obtained from Charles River Laboratories (Wilmington, MA). All mice were given food and water *ad libitum*.

Wounding

For excisional wounds, we used a “humanized” excisional wound model that used silicone stents to force wounds to heal by re-epithelialization more than contracture¹⁵. Briefly, 8-week old C57Bl/6 mice were anesthetized and aseptically prepped prior to creation of two 6 mm full-thickness excisional wounds on the dorsum. An 8 mm diameter circular silicone stent (Sigma-Aldrich; Saint Louis, MO) was placed around the perimeter of the wound and was secured to the skin with Krazy Glue (Toagosei Co, Tokyo, Japan) and interrupted sutures using 6-0 Nylon. Wounds were covered with sterile Tegaderm (3M Healthcare, St. Paul, MN). 3,6-DMAD (obtained from the compound library at Auckland Cancer Society Research Centre at University of Auckland, New Zealand) was dissolved in dimethyl sulfoxide (DMSO) and topically applied to the wound at indicated times in different concentrations with DMSO alone as control. Digital photographs were taken daily until wounds completely epithelialized. The un-epithelialized wound area was quantified using Photoshop software (Adobe Systems, Inc., San Jose, CA) and expressed as a percentage of the original wound.

For the hypertrophic scar model, we used a previously described distraction model¹⁶. Briefly, 8-week old C57Bl/6 mice were prepped for surgery as described above. A 2 cm dorsal midline incision was made with a scalpel on the mouse. The incision was immediately approximated using 6-0 Nylon suture. Biomechanical distraction devices were constructed from 22-mm expansion screws (Great Lakes Orthodontic Products, Tonawanda, NY) and Luhr plate supports (Stryker-Leibinger Co, Freiburg, Germany). On post-operative day (POD) 5, the mice were re-anesthetized, the suture removed, and the distraction devices placed with the bases resting on either side of the incisional scar. Distraction devices were secured in place with KrazyGlue (Toagosei Co, Tokyo, Japan) and 6-0 Nylon suture. Tension was applied to the incisional scar via rotation of the screws on the device to achieve 2 mm of distraction daily. Mice were treated with DMSO or 300 μ M 3,6-DMAD daily from the beginning of distraction until harvest. Wounds were distracted for 2 weeks after which the mice were sacrificed and the dorsal skin, including the scar tissue was harvested and processed for histology as described below.

Luciferase Assay

Relative XBP1 splicing activity was assayed by quantifying luciferase activity using the IVIS Spectrum imaging system (Perkin Elmer, Waltham, MA). The *Xbp1-Luc* transgenic mice were injected intraperitoneally with luciferin (300 mg/kg dissolved in PBS). Mice were anesthetized with isoflourane gas for imaging. Exposure times ranged from 10 seconds to 1.5 minutes and *in vivo* bioluminescent signals were analyzed using Living Image software

(Caliper Corporation, Newton, MA). Bioluminescent signals were quantified as photon count per second per cm² and were normalized to background uptake in the remainder of the animal.

Histological Analysis

Specimens were harvested and fixed in 4% paraformaldehyde for 12 hours and embedded in paraffin. 10 µm sections were made and stained with hematoxylin and eosin. Photographs were taken with a Leica microscope (Leica Microsystem, Solm, Wetzlar, Germany) and scar thickness was analyzed with Adobe Photoshop CS2 (Adobe Systems, Inc., San Jose, CA).

For histomorphometry, we made 10 µm sections throughout the length of the 2 cm hypertrophic scar and every 15th section was used for scar measurement. A total of 40 sections representing the central 1 cm of the scar were analyzed per hypertrophic scar and 4 scars were analyzed per experimental group. Scar volume was calculated for each slide and the volume of scar was calculated for each hypertrophic scar.

Isolation and Primary Culture of Mouse Fibroblasts

Dorsal skin was harvested from 5-day old C57Bl/6 mice. The skin was minced thoroughly with a scalpel and underwent digestion with Collagenase A (Roche Applied Science, Indianapolis, IN) for 60 minutes at 37°C. Following centrifugation, the cell pellet was washed with DMEM (Life Technologies, Rockville, Maryland) and strained through a 100 µm filter. The fibroblast cells were then seeded onto a T75 culture flask and were maintained in media consisting of DMEM with 10% fetal bovine serum, 1% penicillin/streptomycin at 37°C, 5% CO₂. Culture media was changed every two days and fibroblasts were used for experiments within the first 3 passages.

Western Blotting

Tissue samples were lysed in RIPA lysis protein extraction buffer (Millipore, Temecula, CA) containing 0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40 and 10mM EDTA; protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL) was added to the lysates. Samples were briefly sonicated and homogenized by shaking at 4° C for 1 hour. Lysates were then vortexed and centrifuged (11,000 RCF) for 15 minutes at 4° C. Next, supernatant samples were separated, quantified using BCA Protein Assay kit (Thermo Scientific, Rockford, IL) and run in a 10% SDS gel and immunoblotted against spliced-XBP1 and β-actin (both Santa Cruz Biotechnology, Dallas, TX). ChemiDoc XRS+ system (Bio-Rad, Hercules, CA) was used to image the protein bands with ECL detection reagent (GE Healthcare, Pittsburgh, PA).

Wound Tensile Strength Testing

Incisional midline dorsal wounds were created 2 cms in length and were allowed to heal fully while receiving either control DMSO or experimental 300 µM 3,6-DMAD treatment daily. On POD 10 the skin was cut out in a dumbbell shape across the healed wound and tensile strength testing was performed using a Microtester (Bionix 200) equipped with a 100 N load cell as previously described¹⁷. The tissue specimens were attached on either side to grips using double-sided tape and screw-in serrated plates. The tissue was stretched until a

break at the healed incision was detected. Tensile strength was determined as the greatest true stress achieved.

Statistical Analysis

Data analysis was performed using Student's T-test with statistical significance assigned for $p < 0.05$. All data are expressed with $\pm$ SD. All experiments were performed at least in triplicate and in two independent experiments.

RESULTS

To establish that IRE1 was activated during wound healing, we used transgenic mice (*Xbp1-Luc*) expressing a construct in which luciferase is fused in-frame with the spliced form of XBP1 (XBP1s)¹⁴. Under non-ER stress conditions, there is little expression of the chimeric XBP1s-luciferase protein. However, under ER stress, *Xbp1* mRNA is spliced, resulting in translation of the XBP1s-luciferase chimeric protein which is readily detectable by bioluminescent imaging of the live animal. We created excisional wounds on the back followed by imaging at various times after wounding. We found that XBP1 activation was detected beginning at 10 days after wounding with a peak at 14–16 days and persistent expression above baseline out to 20 days (Figure 1). This observation correlates with our hypothesis that IRE1 activity would be greatest during the proliferative phase of wound healing when protein synthesis peaks due to increased proliferation and deposition of new matrix in the wound bed.

We used a small molecule inhibitor of IRE1, 3,6-DMAD, to examine whether inhibition of IRE1 activation had any impact on wound healing. This molecule was identified through the XBP1s-luciferase reporter-based chemical library screen using HT1080 human fibrosarcoma cells and was also shown by computational docking to interact with the RNase domain of IRE1¹³.

We empirically tested an array of concentrations of 3,6-DMAD *in vivo* in mice. We used a “humanized” model of mouse wound healing where the excisional wound is stented open by being sutured to a silicone ring¹⁵. Normally in mice, the majority of wound healing is by contracture and this stenting forces the wound to heal more by re-epithelialization with kinetics of wound closure that reflect human excisional wounds. Mice were treated daily after wounding and the time to total wound closure was recorded. At the completion of healing, the scar was excised for histology.

We found that treatment with concentrations up to 300 μ M 3,6-DMAD led to no difference in time to complete wound closure (Figure 2). We examined the histology of excisional scars of mice treated with 3,6-DMAD. Multiple slides in each excisional wound were measured for thickness to ensure there was no sampling error, and then combined with the data from multiple mice. These data showed a progressive decrease in scar thickness with an increase in concentration used ($p = 0.326$ for 1 μ M, and < 0.05 for all other concentrations compared to control DMSO, $n = 8$ for each concentration) (Figure 3). Since 300 μ M did not delay wound healing and showed the greatest impact on scar thickness, we performed all further experiments with this concentration.

To confirm that we were getting appropriate inhibition of *Xbp1* splicing, we treated excisional wound on POD12, when XBP1 activity is at its peak, with 300 μ M 3,6-DMAD and collected tissue 1 and 2 hours following treatment. A protein extract was made from the treated wounds and the presence of XBP1s was assessed by immunoblot. This demonstrated appropriate inhibition with over 50% less XBP1s present at both time points (Figure 4).

Since we found decreased scar thickness in this excisional wound model, we decided to test the effects of 3,6-DMAD on a mouse model of hypertrophic scarring¹⁶. In this model, mechanical distraction was begun on POD5 of an incisional wound that was closed. Daily distraction placed regular mechanical stress on the wound resulting in a thickened scar. Mice were treated with either 300 μ M 3,6-DMAD or DMSO for control beginning on the first day of distraction and then daily for two weeks until the scar was harvested. To more accurately assess scar volume, we performed histomorphometry across the central 1 cm of the scar. The same number of sections was analyzed in each animal and the area of scar calculated per section. These areas were then summed to derive the total area of scar in each mouse. This demonstrated that 3,6-DMAD-treated mice had significantly smaller scar areas of $19.84 \pm 4.41 \text{ mm}^2$ versus $34.92 \pm 6.52 \text{ mm}^2$ in DMSO-treated wounds, $p < 0.01$, $n = 4$ (Figure 5).

Due to concerns over reduced scar formation potentially causing reduced strength in wounds, we performed tensile strength testing on incisional wounds which were treated with control DMSO or experimental 300 μ M 3,6-DMAD treatment and no difference in wound tensile strength was seen with peak stresses of $0.55 \pm 0.17 \text{ MPa}$ versus $0.40 \pm 0.24 \text{ MPa}$, respectively ($p = 0.3559$, $n = 4$, Figure 6).

DISCUSSION

The UPR is recognized as an important adaptive response within cells to increased protein synthesis. It serves to help cells adapt to increased demands on the ER and Golgi during these periods, or leads to apoptosis if cells remain persistently stressed^{5,8,9}. Wound healing represents a well-defined process where high rates of protein synthesis are required in order to repair the skin wound. While it seems intuitive that the UPR is up-regulated during wound healing, it had yet to be demonstrated. Furthermore, we hypothesized that inhibition of UPR activation, specifically of IRE1, might inhibit wound healing and point towards modulation of the UPR in the treatment of wound healing pathologies from chronic wounds to excessive scarring as is seen in keloids.

We demonstrate here that IRE1/XBP1 was up-regulated during wound healing. Using a *Xbp1-Luc* reporter mouse, we observed activation of XBP1 beginning at 10 days after wounding, and persisted to 20 days with a peak activation at 14–16 days. This peak expression correlated well with the proliferative phase of wound healing during which fibroblast proliferation, migration and deposition of new ECM components were maximal, but we also noted persistent expression above baseline for at least a week following full re-epithelialization. Wounds are known to continue to be sites of active proliferation and matrix synthesis during the period of scar remodeling, and we speculate that persistent XBP1 activation was related to this process. While this time course correlates well with matrix deposition, it also overlaps with a number of other processes occurring during wound

healing including inflammation and angiogenesis, and it is equally possible that IRE1 inhibition affected them to result in decreased scar formation as further discussed below.

While these studies demonstrated that XBP1 was activated during wound healing, it did not necessarily indicate its importance in the process. To show this, we treated wounds with 3,6-DMAD, a highly specific small molecule inhibitor of IRE1. There was a dose-responsive effect on the rate of wound healing in topically treated mice. Mice treated with 300 μ M 3,6-DMAD healed excisional wounds in the same time as controls, but histology demonstrated significantly thinner scars. Finally, we examined a mouse model of hypertrophic wounds and showed treatment led to a significant decrease in scar. Treatment of incisional wounds with 3,6-DMAD did not compromise wound strength. We concluded that XBP1 activation correlated with the proliferative phase of wound healing, and treatment with a specific inhibitor led to decreased scar formation suggesting IRE1/XBP1 have a significant role in wound healing.

One of the things that we did not address in these experiments was the potential role of other pathways of UPR signaling in wound healing. For example, PERK plays a role in generally decreasing translation, and compensatory feedback from blocking XBP1 may also affect PERK, which could have an impact on wound healing¹⁸. In addition to the specific endonuclease activity of IRE1, it also contributes to the IRE1-RIDD pathway, which has been shown to play a part in the decay of ER-localized mRNA^{19,20}. It is possible that RIDD may play some role in the phenotype we see. In experiments examining the effect of 3,6-DMAD on the half-life of *Xbp1* and non-specific RNAs, levels of *Xbp1* mRNA declined, but not the non-specific RNAs, suggesting that RIDD was not affected by 3,6-DMAD (data not shown). Further mechanistic studies on how IRE1/XBP1 affect wound healing are needed, but these experiments do indicate that IRE1/XBP1 plays a major role in wound healing and scar formation.

An important question raised by our data is the cell type affected by 3,6-DMAD. We have shown that treatment with an inhibitor of IRE1 resulted in wound healing with decreased scar formation. However, scarring can be related to fibroblasts production of collagen or immune cell activation, among many possibilities. IRE1/XBP1 is ubiquitously expressed in all cell types, and we know increased protein production will trigger its activation. It is highly possible that any or all of the cells demonstrating increased protein production in a wound will have activation of XBP1. Because wound healing is a complex process involving so many cell types, dissecting the specific effects of 3,6-DMAD will require extensive experimentation and the inability to effectively localize the cell types most responsible for the action of 3,6-DMAD represents a deficit in our data. However, we do think these data represent the one of the first demonstrations of the importance of IRE1/XBP1 activation in wound healing.

Studies of UPR activation in normal tissues have shown it to be most important in specialized secretory cells such as the pancreatic beta cells or B lymphocytes²¹. While fibroblasts in unwounded skin do not produce many proteins, their role changes significantly following wounding. During the proliferative phase of wound healing, these cells need to replace the cellular mass and extracellular matrix defect created by the wound. The

fibroblast does so by proliferating and depositing new matrix, which require significantly increased protein synthesis. Keratinocytes are also required to proliferate to re-epithelialize the wound, but our findings that 3,6-DMAD could reduce scar formation without impacting wound closure suggests fibroblasts are preferentially targeted over keratinocytes in the wound.

There is little data published showing a role for XBP1 in wound healing and hypertrophic scarring. Earlier, we had described increased presence of XBP1 in keloids, a form of pathologic wound healing characterized by persistent deposition of matrix long after the wound has healed¹². This is consistent with our observation that inhibiting XBP1 activity decreased hypertrophic scar formation. More recently, poor wound healing in diabetic mice has been associated with prolonged and excessive activation of the UPR.²² There are other fibrotic processes similar to wound healing such as cirrhosis of the liver and pulmonary fibrosis. In both instances, there have been data to suggest that activation and dysregulation of the UPR may contribute to these diseases.^{23–25} In all of these instances, the data suggest the role of the UPR was complex with activation required as part of the response to injury, but pathology likely related to excessive or persistent activation. XBP1 activity has also been shown to be increased in a number of malignancies associated with the formation of a fibrotic stroma such as pancreatic or breast cancers^{26–28}.

There is clearly much that remains unknown about the role of the UPR and IRE/XBP1 in wound healing. These data, to our knowledge, are the first to show a direct impact of IRE/XBP1 inhibition on wound healing and scar formation. Further dissection of their roles can help tailor drugs targeted for specific pathologies.

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LIST OF ABBREVIATIONS

UPR	Unfolded Protein Response
IRE1	Inositol-Requiring Enzyme 1
XBP1	X-box Binding Protein 1
ATF6	Activating Transcription Factor 6
PERK	Protein Kinase RNA-like Endoplasmic Reticulum Kinase
3,6-DMAD	3,6-dimethyl acetylenedicarboxylate
DMSO	Dimethyl Sulfoxide
RIDD	Regulated IRE-1 Dependent Decay
DMEM	Dulbecco's Modified Eagle's Medium

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Figure 1.

Xbp1-Luc mice (n=5) underwent excisional wounding of the dorsal skin followed with IVIS imaging at the times noted on the x-axis. Expression in the wound was normalized to baseline uptake seen in the remainder of the body. Expression of luciferase was significantly different from baseline (*p<0.05) at all time points after 8 days post-wounding.

Figure 2.

(A) Wound healing curve showing the effect of various concentrations of 3,6-DMAD on mouse excisional stented wounds *in vivo*. Average time to complete healing was not increased for the treatment groups. N=10 wounds per group. (B) Photographs of excisional stented dorsal mouse wounds treated with various concentrations of 3,6-DMAD.

Figure 3.

Impact of 3,6-DMAD on scar thickness. Excisional stented wounds were treated with DMSO or the indicated concentrations of 3,6-DMAD. After complete healing at 12 days, scars were harvested, sectioned and stained. Average scar thickness was quantified as indicated above the graph and representative photomicrographs are shown (x2.5, Bar =100 μm). * $p < 0.05$, $n=8$ for each concentration.

Figure 4.

Western blot performed for presence of XBP1s at 1 and 2 hours post treatment of POD 10 excisional wounds with 300 μ M 3,6-DMAD, β -Actin used for control. Percentage decrease in XBP1s based upon densitometry is noted below. This experiment was performed in triplicate with consistent results.

Figure 5.

(A) Representative histology of hypertrophic scars created in mice. Wound were treated with DMSO 300 μ M 3,6-DMAD as indicated. Highlighted area represents scar area counted. Magnification x2.5, bar =100 μ m. (B) Quantification of scar thickness of hypertrophic scars

treated with control (DMSO) and 300 μ M 3,6-DMAD. *p<0.01, n=4.

Figure 6. Tensile strength testing of incisional wounds treated with DMSO or 300 μ M 3,6-DMAD ($p=0.3559$, $n=4$).