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Unfolded Protein Response Regulation in Keloid Cells

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Abstract

Introduction—Keloids are a common form of pathologic wound healing characterized by excessive production of extracellular matrix. The unfolded protein response (UPR) is a cellular response to hypoxia, a component of the wound microenvironment, capable of protecting cells from the effects of over-accumulation of misfolded proteins. Since keloids have hypersecretion of extracellular matrix, we hypothesized that keloid fibroblasts (KFs) may have enhanced activation of the UPR compared to normal fibroblasts (NFs).

Methods—KFs and NFs were placed in a hypoxia chamber for 0, 24, and 48 hrs. We also used tunicamycin to specifically up-regulate the UPR. UPR activation was assayed by PCR for xbp-1 splicing and by immunoblotting with specific antibodies for the three UPR transducers. Nuclear localization of XBP-1 protein in KFs was confirmed by immunofluorescence.

Results—There is increased activation of XBP-1 protein in KFs compared to NFs following exposure to hypoxia. PERK and ATF-6, two other pathways activated by the UPR, show comparable activation between KFs and NFs. We confirmed that there is enhanced activation of XBP-1 by demonstrating increased nuclear localization of XBP-1 using immunofluorescence.

Conclusion—In contrast to our initial hypothesis that keloids would have broad activation of the UPR, we demonstrate here that there is a specific up-regulation of one facet of the UPR response. This may represent a specific molecular defect in KFs compared to NFs, and also suggests modulation of the UPR can be used in wound healing therapy.

Keywords

Keloids; Unfolded Protein Response; Wound Healing; Fibroblasts

INTRODUCTION

Keloids are a form of pathologic wound healing afflicting a large segment of our population without a definitive therapy. Any patient may develop them, but dark skinned ethnicities are more susceptible to keloid formation with upwards of 15% of the population suggested to be

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at a heightened risk.^{1–5} Despite a wide array of therapeutic techniques, the rate of recurrence following excision remains high, suggesting that the underlying abnormalities have yet to be fully elucidated.¹ Because keloids are the result of the excessive production of extracellular matrix (ECM), we hypothesized that fibroblasts from keloids can demonstrate differences in the unfolded protein response (UPR) compared to normal fibroblasts.

The endoplasmic reticulum (ER) is the principle site for the proper folding and maturation of proteins in eukaryotic cells.^{6–9} Because of this, there is more abundant ER in the cells of organs that perform major synthetic functions such as hepatocytes and pancreatic acinar cells. Additionally, the ER provides calcium storage for the cell, astutely responding to various hormones, growth factors, and other stimuli that may alter cellular energy levels.¹⁰ Events that alter ER homeostasis can lead to accumulation of misfolded proteins, which can disrupt cellular function.

The UPR serves to protect the cell from this by affecting cellular transcription and translation to counteract the cellular stresses and to prevent apoptosis, although under more severe or prolonged stress, it can trigger cellular apoptosis.^{10–12} The UPR activates intracellular signaling pathways via three transmembrane proteins present on the ER membrane: IRE1, pancreatic ER kinase (PERK), and activating transcription factor 6 (ATF6).^{6, 13, 14} The resulting signaling leads to diverse transcriptional responses depending upon the tissue type, but are believed to be generally protective. Clinically, dysregulation of the UPR has been implicated in a number of diseases including α 1-antitrypsin deficiency, Parkinson's disease, diabetes, tissue ischemia, and cancer.^{15, 16}

The role of UPR dysregulation in tumorigenesis stimulated us to examine its possible role in keloid pathophysiology. Although they lack malignant potential, keloids do behave as benign dermal fibroproliferative tumors with local recurrence and persistent growth over time.^{4, 17–19} Hypoxia is a well-established inducer of the UPR, and the wound environment in which keloids form is hypoxic.^{6, 20} The purpose of this study is to determine if there is significant difference in the activation of the UPR in keloid fibroblasts (KFs) compared to normal fibroblasts (NFs). We hypothesized that KFs have an aberrant UPR response relative to NFs when exposed to a hypoxic environment.

MATERIALS AND METHODS

Cell Culture

Keloid fibroblasts (KFs) and normal fibroblasts (NFs) were obtained as previously described.⁵ All specimens were obtained following informed consent and with Human Subjects IRB approval from Stanford University in accord with the ethical standards of the Helsinki Declaration of 1975. NFs and KFs were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 2mM glutamine, and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin sulfate) in a 5% CO₂, 95% air incubator at 37° C. Passages 5 thru 8 were used for this study. HT1080 fibrosarcoma cells cultured under the same conditions served as our positive control. NFs and KFs were grown to 70–80% confluence then serum starved with DMEM supplemented with 0.5% FCS for 48 hours to establish quiescence. Cells were placed in a hypoxia chamber (Sheldon Corp., Cornelius, OR) with pO₂ levels < 0.02% for 0, 24, and 48 hrs. As a control, another group of cells from each line remained in the standard incubator for the same period of time. A final group of cells from each line were stimulated with 4 μ g/ml tunicamycin, a known chemical inducer of the UPR.

Immunoblotting

Cells were lysed with RIPA buffer [50 mM Tris Cl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate 0.1% SDS] with protease inhibitor cocktail (Sigma-Aldrich, St.

Louis, MO) and quantified using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein were electrophoresed on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA).

Membranes were probed with primary antibodies directed against the activated form of XBP-1 (Biolegend, San Diego, CA), ATF6 (Imgenex, San Diego, CA), and phosphorylated-PERK (Biolegend, San Diego, CA). Detection was performed by exposure to the appropriate horseradish peroxidase-linked secondary antibody (GE Healthcare UK Limited). Protein bands were then visualized using the chemiluminescence Western blotting substrate (Boehringer Mannheim, Indianapolis, IN) and detected radiographically using Kodak X-OMAT film (Eastman Kodak Company, Rochester, NY). Relative protein expression was determined by densitometry and an image analysis program (ImageJ, NIH, Bethesda, MD). Expression of each protein was normalized to β -actin. All experiments were performed in triplicate.

PCR

Total RNA was harvested by Absolute RNA microprep Kit (Stratagene, La Jolla, CA). RNA was quantified by spectrophotometry and equal amounts used for all samples. Following reverse transcription, the products were amplified with PCR using forward and reverse primers and Taqman PCR Master Mix (Applied Biosystem, Foster City, CA). The primers for XBP-1 were designed based upon GenBank sequences. The sequences of the primers are as follows: Upstream, (Forward XBP-1) 5'-AGC CAA GGG GAA TGA AGT GA-3' and downstream, (Reverse XBP-1) 5'-GGG GAA GGG CAT TTG AAG AA-3'. Amplified DNA products from RT-PCR were then separated on a 3% agarose gel.

Immunofluorescence

KFs and NFs were grown on glass coverslips, incubated under hypoxia or normoxia as above, fixed in 4% formaldehyde, and permeabilized with 0.6% Triton X100 (Sigma-Aldrich, St. Louis, MO). The slides were then incubated with primary antibody directed against activated XBP-1 (Biolegend, San Diego, CA) followed by Alexa Fluor 488 conjugated secondary antibody. Images were taken with an Axioplan 2 microscope with digital camera (Zeiss, Thornwood, NY).

Trypan Blue Cell Survival Analysis

After treatment with 4 μ g/ml of tunicamycin, trypan-blue stain 0.4% (Invitrogen, Grand Island, NY) was used to determine the number of dead cells in NFs and KFs. Dead cells were identified due to the color change of the cells after trypan blue staining. Unstained cells were counted as live. Both live and dead fibroblasts were counted using a hemocytometer.

RESULTS

Xbp-1 splicing following hypoxia in KFs and NFs

Following activation of the UPR, transcriptionally active XBP-1 protein is produced through splicing of the *Xbp-1* mRNA. This splicing event leads to excision of a 26 bp fragment and a change in the open reading frame of the protein.²¹ The resulting XBP-1 protein is 50 kDa compared to 30 kDa for the protein product from the unspliced mRNA, and is a potent transcription factor. We wished to compare activation of XBP-1 in KFs compared to NFs. *Xbp-1* primers that spanned the splice site were used for PCR analysis. The resulting PCR fragments could be resolved on a 3% agarose gel to determine if splicing were occurring. Tunicamycin, an inhibitor of N-glycosylation, and hypoxia were used induce the UPR. These data showed appropriate splicing of *Xbp-1* mRNA in both the NFs and KFs when

stimulated by tunicamycin (Fig. 1A) or after exposure to hypoxia (Fig. 1B) for the indicated times.

Activation of protein mediators of the UPR

The PCR data confirmed up-regulation of the UPR in KFs and NFs, but not in a quantitative manner. To specifically address production of activated protein, we used immunoblotting of the various protein mediators of the UPR. When KFs and NFs were treated with tunicamycin, there was no difference in the production of activated XBP-1 (aXBP-1) protein (Fig 2A). However, when KFs and NFs were placed in a hypoxic environment, there was a relative increase in aXBP-1 protein in KFs compared to NFs that reached statistical significance after 48 hrs (*p = .004) (Fig. 2B).

In addition to XBP-1, PERK and ATF-6 represent the other major mediators of the UPR. PERK is activated by phosphorylation and we immunoblotted the same cell lysates from KFs and NFs exposed to tunicamycin or hypoxia for phosphorylated PERK (P-PERK). In contrast to what was seen with aXBP-1, we saw no difference in levels of P-PERK in KFs and NFs after exposure to either tunicamycin or hypoxia (Fig. 3) suggesting an equal NF and KF response in this signaling pathway. ATF6 is activated by proteolytic cleavage by the UPR and immunoblotting for the activated form also did not show any difference between KFs and NFs after treatment with tunicamycin or hypoxia, similar to what was seen with PERK (data not shown).

We have previously determined that there is no difference in KF and NF survival under hypoxia. To ensure that any differences we saw in activation of XBP-1 was not due to tunicamycin toxicity in KFs relative to NFs, both cell lines were stained with trypan blue to assess cell survival. We found no difference in cell survival after tunicamycin treatment (Fig. 4).

Immunofluorescence

When the activated form of XBP-1 is produced after activation of the UPR, it is translocated to the nucleus to function as a transcription factor. As another method to demonstrate increased activation of XBP-1, we used immunofluorescence to demonstrate increased nuclear localization following hypoxia. NFs and KFs were cultured under normoxic or hypoxic conditions for 48 hrs and then assayed by immunofluorescence using antibody to XBP-1. After culturing NFs and KFs under normoxia for 48 hrs, the vast majority of the staining is present in the cytoplasm (Fig. 5A, C). However, when cultured for 48 hrs under hypoxia, we see markedly increased fluorescence in the nuclei of KFs compared to NFs (Fig. 5B, D). Although immunofluorescence is not a highly quantitative assay, we do see a significant difference in the fluorescence pattern that is consistent with increased activation and nuclear translocation of XBP-1, and with the results from our immunoblot analysis.

DISCUSSION

The UPR has varied functions depending upon cell type. It is most active in what have been termed, "professional secretory cells." These are cells that have a highly active protein translation machinery and include hepatocytes, adipocytes, oligodendrocytes, and pancreatic β -cells.^{10, 15, 22} With the increase in protein translation comes increased amounts of improperly folded proteins that can have a negative impact on cellular function. The UPR is up-regulated in these cells as a protective mechanism and its dysregulation has been implicated in a number of diseases including neurodegenerative disease, obesity, diabetes, and cancer.^{6, 10, 15}

The role of the UPR contributing to the pathogenesis of various forms of cancer led to our interest in its possible role in keloid pathogenesis. Malignant tumors have a tremendous capacity to aggressively divide despite residing in an environment depleted of nutrients and oxygen. It has been noted that despite tumor cells' physiologic barriers to cell survival, there is a paradoxical drive in malignant progression. Interestingly, it appears as if they are better adapted to carryout cell proliferation despite significant stress.⁶ There has been a great deal of investigation into the role of the UPR in tumorigenesis.^{6, 23, 24} These data suggest that there is up-regulation of the UPR as a adaptive response to allow tumors to survive in this relatively nutrient- and oxygen-poor environment.^{6, 25, 26} Although they lack malignant potential, keloids share many characteristics including persistent growth and the tendency to recur after excision, and are recognized as benign dermal fibroproliferative tumors.^{1, 4, 17-19}

We describe here a differential response of the UPR to hypoxia in KFs compared to NFs. Our PCR data shows that there is activation of the UPR in both KFs and NFs, but this was not done in a quantitative manner. Immunoblotting directed against the activated form of XBP-1 confirmed that there is increased protein present in KFs. We demonstrated that the increased UPR activation we see was specifically due to up-regulation of XBP-1 activation, and there was no effect on PERK or ATF6 activation. Furthermore, we show that there was a specific contribution from hypoxia that leads to this increase; treatment with tunicamycin, an activator of the UPR through inhibition of glycosylation, does not lead to increased XBP-1 activation. Our findings suggest that the UPR is indeed heightened in KFs compared to NFs and the possibility of it serving as link between the aforementioned aberrant signaling pathways in KFs, deserves further consideration (Fig. 6).

Analysis of KFs have revealed significant up-regulation of a variety of cellular stress signaling pathways including p38 kinase²⁷, FAK/ERK^{28, 29}, c-Jun N-terminal kinase (JNK)^{30, 31} and NF- κ B.³²⁻³⁴ A common upstream link between these various aberrant signaling pathways involved in keloid pathogenesis have yet to be ascertained. The UPR represents a more proximal cell response to stress and is capable of up-regulating several of the signaling pathways mentioned including both JNK and p38 kinase.

There is evidence that KFs have key pathways that are altered when compared to NFs during a number of stress responses. The UPR is a stress response pathway that has been found to be up-regulated in professional secretory cells, and may have a role in keloid's abnormal stress response signaling. What is not demonstrated by our data is whether the increased activation of IRE-1/XBP-1 is a causative event in keloid formation or secondary to the hypoxic environment of the keloid. Future experiments will use direct agonists of IRE-1/XBP-1 to see if NFs can be made to behave like KFs as a way to address this question.

Another question that is not addressed by this study is when IRE-1/XBP-1 might be up regulated during keloid formation. This would also help in determining whether its up regulation is causative or a consequence of keloids. If IRE-1/XBP-1 is causative, it might be expected that we would see increased activity very early in keloid formation. Unfortunately, we do not have access to keloid specimens timed from their date of onset, but it would be an intriguing experiment to perform if specimens were available.

Many wound healing researchers, including us, believe that keloids are a distinct clinical condition from hypertrophic scars. Although the initial appearance may be similar, the behavior is significantly different with hypertrophic scars limited to the original scar area and spontaneously resolving over time, but with keloids showing progressive growth over time beyond the original scar area. It would be equally informative to study UPR regulation in hypertrophic scars. Our prediction would be that there is no difference between NFs and fibroblasts from hypertrophic scars, but the experiments need to be done to demonstrate this.

There continues to be a clear difference between normal and keloid fibroblast biology that was once again displayed with our UPR data. Continuing the evaluation of stress response signaling in normal and keloid fibroblasts will be imperative to further define keloid behavior and to establish possible targets for more effective therapeutic interventions.

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Figure 1.

Alternative splicing of *xbp-1* is induced both in the keloid (KF) and normal fibroblasts (NF) after stimulation with tunicamycin (**A**) and hypoxia exposure (**B**). Unspliced *xbp-1* (U-XBP1) is detected at ~260bp, while spliced *xbp-1* (S-XBP1) is detected at ~230bp. (Hyp= Hypoxia, Nor= Normoxia)

Figure 2.

(A) No statistically significant difference seen in activated XBP-1 (aXBP1) protein expression upon western blot analysis of KFs and NFs after 6, 12, and 24 hours (hrs) of tunicamycin (TM) treatment. (B) KFs reveal a two fold increase in aXBP-1 protein expression relative to NFs after 48 hrs of hypoxia exposure (* = $p < .05$). Controls (+C) for aXBP-1 expression are hypoxia exposed HT1080 fibrosarcoma cells.

Figure 3.

(A) No statistically significant difference seen in phosphorylated PERK (P-PERK) protein expression upon western blot analysis of KFs and NFs after 6, 12, and 24 hours (hrs) of tunicamycin (TM) treatment. (B) Similarly, there was no statistically significant difference displayed in P-PERK protein expression in KFs compared to NFs after 24 or 48 hrs of hypoxia exposure. Controls (+C) for aXBP-1 expression are hypoxia exposed HT1080 fibrosarcoma cells.

Figure 4.

(A) Trypan blue staining to assess cell survival revealed no statistically significant difference in cell death when comparing the KFs and NFs after 6, 12, and 24 hours (hrs) of tunicamycin treatment. (B) Similarly, trypan blue staining to assess cell survival after 24 and 48 hrs of normoxia and hypoxia revealed no statistically significant difference in cell death when comparing KFs and NFs.

Figure 5.

Immunofluorescence for aXBP1 of NFs reveals minimal signaling after 48 hours of normal incubation (**A**) and moderate signaling and nuclear translocation after 48 hours of hypoxia exposure (**B**). There is minimal aXBP1 signaling in KFs after 48 hours normoxia (**C**). In contrast to NFs, there is relatively heightened aXBP1 signaling within the nuclei of KFs upon 48 hours of hypoxia exposure (**D**).

Figure 6.

Schematic of the activated Unfolded Protein Response (UPR) signaling cascade. After hypoxia exposure, misfolded proteins in the lumen of the endoplasmic reticulum lead to the activation of the three UPR transducers pancreatic ER kinase (PERK), inositol-requiring-1 α /X-box binding protein-1 (IRE1/XBP1), and activating transcription factor-6 (ATF6). Upon hypoxia incubation, (A) NFs have relatively less activation of IRE1/XBP1 then (B) keloid fibroblasts (thick arrow).