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Inherent Differences in Keratinocyte Function in Hidradenitis Suppurativa: Evidence for the role of IL-22 in disease pathogenesis

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Abstract

Hidradenitis suppurativa (HS) is a chronic, recurrent, inflammatory disease of apocrine gland-bearing skin which affects approximately 1–4% of the population. Defective keratinocyte function has been postulated to play a role in HS pathogenesis. Using an in-vitro scratch assay, differences between normal, HS and chronic wound (CW) keratinocytes were evaluated. Normal keratinocytes exhibited faster scratch closure than HS or CW, with normal samples showing 93.8% closure at 96 hours compared to 80.8% in HS ($p=0.016$) and 71.5% in CW ($p=0.0012$). Keratinocyte viability was similar in normal and HS ($91.12\pm6.03\%$ and $86.55\pm3.28\%$ respectively, $p=0.1583$), but reduced in CW ($72.34\pm13.12\%$, $p=0.0138$). Furthermore, apoptosis measured by annexin V/Propidium iodide, was higher in CW keratinocytes ($32.10\pm7.29\%$ double negative cells compared to $68.67\pm10.37\%$ in normal and $55.10\pm9.46\%$ in HS, $p=0.0075$). Normal keratinocytes exhibited a significantly higher level of IL-1 α (352.83 ± 42.79 pg/ml) compared to HS (169.96 ± 61.62 pg/ml) and CW (128.23 ± 96.61 pg/ml, $p=0.004$). HS keratinocytes exhibited significantly lower amounts of IL-22 (8.01 pg/ml) compared to normal (30.24 ± 10.09 pg/ml) and CW (22.20 ± 4.33 pg/ml, $p=0.0008$), suggesting that defects in IL-22 signaling may play a role in HS pathogenesis. These findings support intrinsic differences in keratinocyte function in HS which cannot be attributed to reduced keratinocyte viability or increased apoptosis.

Keywords

Wound; Hidradenitis Suppurativa; Keratinocyte; IL-22; IL-1 α ; VEGF

INTRODUCTION

Hidradenitis suppurativa (HS) is a chronic, recurrent, inflammatory disease of the apocrine sweat glands, characterized by recurrent abscessing inflammation which affects

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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approximately 1–4% of the population ^{1,2}. There is currently no known cure for HS and the pathogenesis is poorly understood. Host innate and adaptive immune responses ³, defective keratinocyte function ⁴, and the microbial environment in the hair follicle and apocrine gland ⁵ have all been postulated to play a role in disease activity. It is known that HS patients develop inflammatory skin lesions with elevated levels of pro-inflammatory cytokines and matrix metalloproteinases ^{6,7}. Furthermore, studies investigating cellular and cytokine responses in HS lesional tissue suggest defects in immune responses with increased numbers of infiltrating CD4+ T cells producing IL-17 and IFN- γ , and reduced numbers of IL-22 secreting cells ⁸. However, there is an unmet need to clarify the role that keratinocytes play in orchestrating the inflammatory responses seen in this disease.

Keratinocytes are epidermal cells that are programmed to maintain integrity of the skin. Tissue injury promotes keratinocyte migration, activation ⁹ and production of cytokines and growth factors ^{10–12}. Normal keratinocyte turnover involves balances in proliferation and apoptosis to maintain normal epidermal thickness ¹³. Alterations in keratinocyte viability and apoptosis are known to play a role in delayed wound healing in patients with chronic wounds ¹⁴, and it has been postulated that accelerated apoptosis may contribute to the pathogenesis of HS.

Parallels have been drawn between the chronic inflammatory state in chronic wounds (CW) and HS inflammation. Normal wound healing involves four overlapping phases that progress sequentially regardless of wound etiology to achieve restoration of the skin barrier function ¹⁵. Many CW are arrested in the inflammatory phase ^{15–17}, and are unable to transition to the proliferative phase with concurrent upregulation of angiogenesis and matrix deposition. Increased apoptosis and reduced cell viability are known to play a role in delayed healing in chronic wounds, particularly in relation to chronic biofilm colonization ¹⁸, and it is thought that these abnormalities arise from defects in keratinocyte function ^{14,19}. Given the chronicity and recurrence of HS lesions, it is reasonable to investigate differences in keratinocyte behavior and function in HS compared to CW.

The in-vitro scratch assay is an established method for investigating keratinocyte function and has been used to measure cell migration and cell-cell interaction ²⁰. This assay has been used to investigate migration of primary keratinocytes and keratinocyte cell lines in response to a scratch stimulus ^{21,22}. A benefit of this system is that it facilitates the study of keratinocytes in isolation, without supporting stromal cells or immune cells which otherwise complicate interpretation of results. Apoptosis and cell viability can be evaluated in this system using flow cytometry and fluorescent staining. Furthermore, cytokine measurement from culture supernatants allows for assessment of cytokine production by keratinocytes in different disease states and time points.

The purpose of the current study was to investigate keratinocyte function in HS by comparing cellular migration, cytokine responses and cell viability of primary cultured keratinocytes isolated from patients with HS, patients with CW and from normal skin. The overarching hypothesis was that HS keratinocytes would demonstrate differences in migration, cell viability and functional cytokine production compared to CW and normal keratinocytes.

METHODS

This research was conducted through the Wound Etiology and Healing (WE-HEAL) Study, a biospecimen and data repository designed for studying CW and HS approved by the George Washington University Institutional Review Board (041408). De-identified HS and CW skin samples were harvested from discarded lesional tissue post-surgical excision. HS samples were obtained from patients with active Hurley stage III disease undergoing surgical excision. Normal skin samples were harvested from discarded abdominoplasty tissue through a protocol approved by the George Washington University Institutional Review Board (101419). For the purpose of this study, primary keratinocytes from HS, CW and normal skin samples were cultured. Three unique samples from each disease group were cultured in four replicates.

Keratinocyte Isolation Protocol

Isolation of keratinocytes from human tissue specimens was performed according to established methods^{23,24}. Tissue specimens were cut into small strips containing dermis and the epidermis, with adipose and connective tissue removed. Specimens were then washed three times in PBS (GE Healthcare Bio-Sciences, Logan, UT) with 1X Penicillin/Streptomycin (Life Technologies, Grand Island, NY). Specimens were immersed in dispase solution (ThermoFisher Scientific, Waltham, MA) in the concentration of 2.5 mg/ml dissolved in EpiLife media (Invitrogen/Cascade Biologics, Eugene, OR) with antibiotics, keeping the dermal side down at 4°C for 12–16 hours overnight. Following overnight digestion, the epidermis was separated from dermis and transferred into 15 mL of TrypLE select (Life Technologies/Gibco, Grand Island, NY). The epidermis was minced and maintained at 37°C for 30–40 minutes. Equal amounts of DMEM (ThermoFisher Scientific, Waltham, MA) supplemented with 10% FBS was added to halt digestion. The solution was passed through a sterile 70-µm filter to remove undigested fragments. The suspension was centrifuged and suspended in EpiLife media with 60 µM calcium supplemented with Human Keratinocyte Growth Supplement (HKGS, Invitrogen/Cascade Biologics, Eugene, OR).

Keratinocyte Culture Protocol

Human epidermal keratinocytes isolated from the skin samples were seeded at 5×10^5 cells per T75 flask (Eppendorf, North America, Hauppauge, NY). Media was changed at 72 hour intervals, and cells took approximately three weeks to achieve confluence. For all cell cultures, EpiLife media was used with HKGS as supplement.

Keratinocyte Scratch Assay

The in-vitro scratch assay was performed according to established protocol²⁰. Keratinocytes were plated in 6-well plates with 2×10^5 cells/well. The media was changed every 3 days until cells achieved 80% confluence. Keratinocytes from each culture line were plated in duplicate, such that each 6-well plate had one sample from each of the three groups in duplicate. This served as internal control since each plate always had all three groups. Duplicate plates were used as an external control. Once confluence was reached, a single scratch was made using sterile 1 mL pipette tip in each well, perpendicular to two reference lines drawn at the bottom of the well²⁵. These reference lines were used for image

alignment throughout the migration assay ensuring a consistent field of view. Images were captured just prior to scratch and then at 0, 24, 48, 72, and 96 hours after the scratch, using an inverted phase contrast microscope (Leica DM-IRB, Leica Microsystems, Buffalo Grove, IL) at 50X magnification. Scratch surface area was measured using ImageJ software (National Institutes of Health, Bethesda, MD).

Cell Viability Assay

Cell viability was assessed at 96 hours after the scratch using the Ready Probes Blue/Green Cell Viability Imaging kit (ThermoFisher Scientific, Waltham, MA). In this assay, the Nuc-Blue reagent is cell membrane permeable, stains the nuclei of all cells and is detected using a standard DAPI filter. The Nuc-Green reagent does not permeate intact cell membranes; it thus stains only the nuclei of non-viable cells in which plasma membrane integrity is compromised, and is detected using a green (FITC/GFP) filter. Images for this cell viability assay were captured using a fluorescence microscope (Nikon Eclipse TE300, Nikon Instruments, Melville, NY) at 100X magnification. The images were merged to extract a composite image of the ratio of viable/non-viable cells. Two images from each well were assessed by two independent observers, blinded as to clinical group. Results were presented as mean percentage cell viability, based on two independent observers for all groups.

Analysis of Apoptosis

Apoptosis was assessed at the 96-hour time-point using the Annexin V/Propidium iodide (PI) apoptosis assay (BioLegend, San Diego, CA). In this assay, early apoptotic cells stain positive for Annexin V only, whereas late apoptotic and necrotic cells are double positive for Annexin V and PI²⁴. Keratinocytes were dislodged from culture wells using TrypLE select. Cells were washed twice in cell staining buffer then re-suspended in the Annexin V binding buffer. Following manufacturer's protocol, 5 µL of the Annexin V and 10 µL of the PI solution were added to 100 µL of the cell solution (2.5×10^6 cells/ml) and incubated for 15 minutes in the dark. Next, 400 µL of the Annexin V binding buffer was added to each tube and the cells were analyzed using Fluorescein Isothiocyanate (FITC) and Phycoerythrin (PE) channels by flow cytometry.

Estimation of Cytokine Production

Samples of the culture supernatants were harvested during the scratch assay from each well at 0, 24, 48, 72, and 96 hours after scratch for cytokine analysis. The Human Magnetic Luminex Screening Assay (Human Premixed Multi-Analyte kit; R&D systems, Minneapolis, MN), using the MAGPIX System (EMD Millipore, Billerica, MA) was used to measure cytokine profile of culture supernatant. Samples were processed in duplicate and results were calculated from the standard curve following manufacturer's protocol. Final concentrations were calculated utilizing Milliplex analyst software 5.1 (EMD Millipore, Billerica, MA).

Statistical Analysis

Statistical analysis was conducted using SAS software, version 9.3 (SAS Institute Inc., Cary, NC). A two-sided P value of less than 0.05 was used to indicate statistical significance. The

values for individual data points were represented as the mean of wells. Scratch closure was measured for each subject at 0, 24, 48, 72, and 96 hours. In order to account for autocorrelation within subjects over time, a random-effects mixed-model was used to examine whether the pattern of change in scratch size over time differed between diagnostic groups. This involved specifying a model with main effects for group and time and a group x time interaction, using the SAS Mixed procedure with the denominator degree of freedom method. A general linear model was used to examine differences in mean percent-change from baseline in scratch surface area across groups, within time periods. Cytokines were measured at 0, 24, 48, 72, and 96 hours.

RESULTS

HS and CW Keratinocytes show Delayed Migration Compared to Normal Keratinocytes

In the keratinocyte scratch assay, keratinocyte migration as evidenced by scratch closure rate was significantly slower in HS keratinocytes compared to normal at 96 hours, and compared to CW at all time points (Figure 1A). We examined the percent change in scratch surface area over time, using a random effects mixed model with standard errors. This allowed comparison of the change in scratch surface area over time, both within subjects and between diagnostic groups. The group-time interaction was used to assess whether mean scratch surface area changed at a different rate over time across the disease groups. There were significant differences in change in mean scratch surface area between all disease groups ($p=0.0076$) and over time ($p<0.0001$, Figure 1A). Additionally, the mean rate of change in scratch surface area over time differed significantly between normal and CW groups (Figure 1B). There were significant differences in mean percent closure between diagnostic groups, with normal subjects having significantly more closure than CW at all time points, and significantly more closure than HS at 96 hours.

HS and Normal Keratinocytes Exhibit Higher Cell Viability Compared to CW

To understand whether differences in cell viability contributed to differences in scratch closure between normal, HS and CW, the Ready Probes Blue/Green Cell Viability Imaging kit was used to measure the percentage of viable cells. The green dye is impermeable to viable cells, which thus only stain blue, while non-viable cells stain green (Figure 2A). The viable to non-viable ratio was compared between diagnostic groups using one-way analysis of variance. Normal (Mean $\pm$ SD, $91.12\pm6.03\%$) and HS ($86.54 \pm 3.28\%$) keratinocytes had a significantly higher percentage of viable cells than CW ($72.3\pm13.1\%$, $p=0.0046$, Figure 2B).

HS and Normal Keratinocytes had Lower Rates of Apoptosis than CW Keratinocytes

To confirm whether the differences in viability were due to differences in apoptosis, Annexin V/ Propidium Iodide (PI) staining was assessed using flow cytometry. Cells negative for Annexin V and PI are viable. Cells positive for Annexin V only represent early apoptotic cells, whereas Annexin V/PI double positive cells are non-viable, late apoptotic cells. The percent of Annexin V/PI double negative (viable) cells was significantly higher in normal ($68.67\pm10.37\%$) and HS ($55.10\pm9.46\%$) compared to CW ($32.10\pm7.29\%$, $p<0.05$). This indicates that CW keratinocytes had higher rates of apoptosis than normal and HS, and

that differences in scratch closure rate seen in HS were unlikely to be secondary to apoptosis (Figure 3A and B).

To further assess susceptibility of keratinocytes to apoptosis, keratinocytes from each group were cultured with 5mM H₂O₂ for 4 hours and then analyzed for Annexin V expression by flow cytometry. Normal keratinocytes had lowest Annexin V expression following H₂O₂ exposure (Figure 3C) indicating that they were the most resistant to apoptosis. The Mean Fluorescence Index (MFI) for Annexin V was significantly higher in CW (44971.33±8610.36) compared to normal (8279.33±341.07) and HS (19519.33±8473.90, $p<0.05$, Figure 3D).

IL-22 is Significantly Lower in HS Compared to CW and Normal Keratinocytes

To investigate keratinocyte cytokine production, culture supernatants collected at each time point were analyzed using the human magnetic Luminex screening assay and the MAGPIX system was used to measure the cytokine profile. For each cytokine, data was analyzed using a mixed model with time effect within each diagnostic group in order to test whether the later time points differed from time 0. A mixed model with group-time interaction was also used to investigate whether the groups changed at different rates.

HS keratinocytes produced significantly lower amounts of IL-22 at all time-points of the assay compared to keratinocytes from normal and CW (Figure 4A, $p=0.0004$, 0.0036, 0.045, and 0.005 for 24, 48, 72 and 96 hours respectively). In contrast, both normal and CW keratinocytes had a steady IL-22 expression with mean concentrations of 30.24±10.09 and 22.20±4.32 pg/ml respectively at 96 hours. Across the groups, IL-22 did not change over time (time effect $p=0.18$) and there was no group-time interaction ($p=0.75$), suggesting that the differences between the groups were stable over time.

VEGF and IL-1 α Release Increased in Normal Keratinocytes in Response to Scratch

As has been reported by others, in response to scratch, normal keratinocytes exhibited increase in Vascular Endothelial Growth Factor (VEGF). Significantly higher concentration of VEGF was observed in normal keratinocytes (1263.32±311.01 pg/ml) at 96 hours compared to HS (402.96±364.13 pg/ml) and CW (411.44±283.84 pg/ml, $p<0.001$; Figure 4B). IL-1- α also increased over time in all groups with normal keratinocytes having a mean concentration of 352.83±42.79 pg/ml at 96 hours which was significantly higher than HS (169.96±61.62 pg/ml) and CW (128.23±96.61 pg/ml, $p=0.0004$; Figure 4C). There was no significant change between groups or over time for TNF- α , IL6, IL3, IL8, EGF, IL1- β , IFN- γ , IL1-RA, IL4, IL17A, IL2, GM-CSF, IL5, G-CSF, IL12p70, IL15, FGF basic or HGF. Although IL10 was significantly higher in the normal compared to HS and CW samples in group-time calculations ($p=0.0363$), the biological values across the groups were not notably different.

DISCUSSION

HS patients are known to have delayed wound healing^{26,27} and prior studies have suggested that intrinsic defects of keratinocytes may play a role in the pathophysiology of HS⁸. Using cultured keratinocytes we were able to demonstrate that HS keratinocytes exhibited slower

scratch closure rate than normal keratinocytes. Since this assay investigates keratinocytes in isolation, without other stimuli and immuno-modulating factors, it is likely that differences in keratinocyte function contribute to the differences in scratch closure seen between normal and HS keratinocytes.

The studies investigating cell viability and apoptosis demonstrated that unlike the differences seen in CW keratinocytes, HS keratinocyte dysfunction could not be attributed to increased apoptosis or reduced cell viability. In contrast, significant differences in IL-22 production were seen at all time points in the HS keratinocytes compared to normal and CW. This provides evidence that IL-22 and its downstream pathways may play a crucial role in keratinocyte dysfunction in HS.

IL-22 is a member of the IL-10 family of cytokines which plays a role in cutaneous innate immunity via stimulation of anti-microbial peptides through a STAT3 dependent pathway^{28–30}. IL-22 also enhances proliferative and anti-apoptotic pathways, and regulates genes responsible for cellular differentiation and mobility³¹. Altered patterns of IL-22 and anti-microbial peptide production have been demonstrated in HS^{8,32–35}, and HS skin lesions have previously been shown to exhibit lower levels of IL-22²⁹. A double-blind, randomized, placebo-controlled trial investigating the use of the IL-1 inhibitor anakinra demonstrated that IL-1 blockade in HS was associated with both improved disease activity and elevation of IL-22 production in anakinra treated patients³⁶. In the current study, cytokine analysis from culture supernatants showed that HS keratinocytes exhibited significantly lower levels of IL-22 production compared to CW and normal. While prior studies have indicated that reduced IL-22 in HS may be a function of reduced cutaneous IL-22 producing CD4+ T cells⁸, the current study investigated cultured keratinocytes in isolation. Demonstration of significantly reduced IL-22 in HS keratinocytes compared to normal and CW in this model suggests that HS keratinocytes may have intrinsic deficits in IL-22 production, further highlighting the role of IL-22 and its downstream pathways in HS.

Of the other cytokines evaluated in this study, both VEGF and IL-1 α increased in response to scratch and were higher in normal keratinocyte supernatants than HS and CW. IL-1 α is a stimulator of keratinocyte migration, independent of other growth factors³⁷, and thus the faster scratch closure rate in normal keratinocytes may well have been secondary to this more robust IL-1 α response. IL-1 α and IL-22 are also reported to cause inhibition of keratinocyte differentiation³⁸, and since keratinocyte differentiation is important in maintaining skin integrity the interplay between these two cytokines merits further study.

Further evidence to support a role for the interplay of IL-1 and IL-22 in HS are the small clinical studies investigating the use of anakinra (Kineret®), a recombinant IL-1 receptor antagonist, in patients with HS. In a double-blind randomized trial³⁹, anakinra efficacy in HS was correlated with cytokine production by peripheral blood mononuclear cells (PBMC), with the placebo arm showing increased IFN- γ production and the anakinra arm demonstrating increased IL-22. Epidermal production of antimicrobial peptides, in particular beta-defensins, has also been shown to be modulated by IL-1 inhibition⁴⁰; further supporting the need for additional investigation of these pathways in HS.

VEGF also increased in response to scratch in all groups, with significantly higher levels in normal keratinocytes. While well recognized as a potent stimulator of angiogenesis, VEGF is an endothelium specific growth factor, both expressed and secreted by epidermal keratinocytes and constitutively produced by cultured keratinocytes⁴¹. VEGF is known to be important in wound repair^{42,43}, and inhibitors of VEGF in clinical use are known to cause major issues with wound healing⁴⁴. The observation that all keratinocyte groups exhibited increased VEGF in response to the scratch stimulus, but that this response was most robust in normal keratinocytes correlating with fastest scratch closure, further supports the integrity of this model.

This study had some important limitations which merit discussion. HS is a complex disease with multiple drivers, including the cutaneous and systemic immune response along with the interplay between the host immune response and the microbiome. In this model we examined keratinocyte function in isolation, without cutaneous or systemic immune responses and in a sterile system without microbial stimuli. However, we were able to demonstrate not only functional differences in keratinocyte migration, but also significant differences in keratinocyte cytokine responses which are biologically congruent with other studies examining HS^{8,36}. Even though the data presented is based on culture of only three human specimens per group, taken together with data from the recent clinical trial of IL-1 blockade in HS³⁶, these findings support a biologic role for defective keratinocyte function in HS and open up new avenues for investigating HS pathogenesis.

In conclusion, this study demonstrates that the keratinocyte scratch assay is a useful and valid model for studying keratinocyte function in HS. Scratch closure was delayed in both HS and CW keratinocytes; however, the delay in HS keratinocytes could not be attributed to differences in keratinocyte viability or apoptosis. Significant differences in IL-22 production in the HS keratinocytes suggest that defective IL-22 production in HS keratinocytes may play a role in HS disease pathogenesis.

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Abbreviations:

HS	Hidradenitis Suppurativa
CW	Chronic Wounds
N	Normal

IL	Interleukin
MFI	Mean Fluorescence Index
FITC	Fluorescein Isothiocyanate
PI	propidium iodide
IFN-γ	Interferon-gamma
VEGF	vascular endothelial growth factor

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Figure 1: HS and CWb keratinocytes show delayed migration compared to normal keratinocytes in response to scratch.

(A) Representative images from the keratinocyte scratch migration assay demonstrate significantly delayed closure in CW and HS samples compared to normal. **(B)** Change in scratch surface area from baseline (% change over time, mean $\pm$ 95% confidence interval). Closure was significantly slower in CW at all time points ($p < 0.05$ between normal keratinocytes and chronic wound keratinocytes at 24, 48, 72 and 96 hours) and slower in HS keratinocytes at the 96 hour time point ($p < 0.05$).

A

B

Figure 2: HS and normal keratinocytes exhibit significantly higher cell viability compared to CW.

(A) Fluorescent microscopy showing viable (blue) and non-viable (green) keratinocytes from normal, HS and CW samples at 96 hours after scratch (magnification=100×.) **(B)** Viable to non-viable ratio was compared between diagnostic groups. Normal and HS keratinocytes had significantly higher percentage of viable cells than CW ($p=0.0046$).

Figure 3: HS and normal keratinocytes had lower rate of apoptosis than CW keratinocytes..

(A) Annexin V/PI staining was assessed using flow cytometry. (B) The percent of annexin V/PI double negative cells was significantly higher in normal ($68.67 \pm 10.37\%$) and HS ($55.10 \pm 9.46\%$) compared to CW ($32.10 \pm 7.29\%$, $p < 0.05$). (C) To assess susceptibility of keratinocytes to apoptosis, keratinocytes from each group were incubated with 5mM H₂O₂ for 4 hours and then analyzed for Annexin V expression using flow cytometry. Normal keratinocytes had the lowest Annexin V expression following H₂O₂ exposure indicating that they were the most resistant to apoptosis. (D) The Mean Fluorescence Index (MFI) for Annexin V was significantly higher in CW (44971.33 ± 8610.36) compared to normal (8279.33 ± 341.07) and HS (19519.33 ± 8473.90 , $p < 0.05$).

Figure 4: Keratinocyte Cytokine Release during Scratch Assay.

(A) IL-22 was significantly lower in HS keratinocytes across all time-points whereas no significant difference was observed in IL-22 secretion over the 96 hour period in normal and CW groups ($p < 0.05$). (B) Normal keratinocytes exhibited increase in VEGF secretion over time with levels significantly higher at 96 hour time-point compared to CW and HS ($p < 0.001$). (C) IL-1 α was significantly higher in normal compared to CW and HS in response to scratch at 72 and 96 hour time-points ($p = 0.0004$).