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Visualization of Morphological and Molecular Features Associated with Chronic Ischemia in Bioengineered Human Skin

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Abstract

We present an *in vitro* model of human skin that, together with nonlinear optical microscopy, provides a useful system for characterizing morphological and structural changes in a living skin tissue microenvironment due to changes in oxygen status and proteolytic balance. We describe for the first time the effects of chronic oxygen deprivation on a bioengineered model of human interfollicular epidermis. Histological analysis and multiphoton imaging revealed a progressively degenerating ballooning phenotype of the keratinocytes that manifested after 48 h of hypoxic exposure. Multiphoton images of the dermal compartment revealed a decrease in collagen structural order. Immunofluorescence analysis showed changes in matrix metalloproteinase (MMP)-2 protein spatial localization in the epidermis with a shift to the basal layer, and loss of Ki67 expression in proliferative basal cells after 192 h of hypoxic exposure. Upon reoxygenation MMP-2 mRNA levels showed a biphasic response, with restoration of MMP-2 levels and localization. These results indicate that chronic oxygen deprivation causes an overall degeneration in tissue architecture, combined with an imbalance in proteolytic expression and a decrease in proliferative capacity. We propose that these tissue changes are representative of the ischemic condition and that our experimental model system is appropriate for addressing mechanisms of susceptibility to chronic wounds.

Keywords

hypoxia; ischemia; ulceration; bioengineered skin; multiphoton; MMP-2

Introduction

Chronic ischemia, a pathology associated with peripheral vascular disease and diabetes, is a significant cause of skin ulceration. Identification of early morphological changes associated with ischemic skin will lead to a better understanding of the molecular mechanisms underlying this condition. A hypothesis recently set forth by Dalton and coworkers (2005, 2007) links inappropriate metabolic processes and elevated extracellular matrix (ECM) turnover with mechanical weakening and susceptibility to proteolytic degradation by enzymes such as MMP-1 and MMP-2, ultimately leading to ulcer formation

Three-dimensional bioengineered models of skin would provide a valuable tool with which to investigate the molecular mechanisms contributing to skin ulceration (Herman & Leung, 2009). We have recently developed a bioengineered model of human interfollicular epidermis that is the basis of our skin microenvironment (SM) model. The SM model consists of an epidermal compartment formed by cells from a human keratinocyte cell line, called NIKS, and a dermal compartment consisting of a gelled type I collagen matrix embedded with human dermal fibroblast cells. The human dermal fibroblasts secrete matrix proteins and growth factors that are necessary for keratinocyte growth and differentiation. When seeded on top of the dermal compartment and cultured organotypically, the NIKS keratinocytes form a fully stratified squamous epithelium (Allen-Hoffmann et al., 2000).

Visual assessment of morphological and molecular features of thick tissues is traditionally achieved with tissue sectioning and standardized histological stains and/or immunohistochemical techniques that require tissue fixation. Noninvasive optical sectioning techniques, including confocal and multiphoton fluorescence microscopy, allow the measurement of structural and biochemical properties of unstained, unfixed living cells and tissues due to endogenous and/or exogenous sources of fluorescence contrast (White et al., 1987; Denk et al., 1990; Centonze & White, 1998; Konig, 2000; Zipfel et al., 2003b; Konig et al., 2006). In confocal microscopy optical sectioning is achieved by the rejection of out-of-focus light by a pinhole aperture that is confocal to the plane of focus at the sample (Pawley, 2006). In multiphoton microscopy, optical sectioning is achieved by generation of high photon-density light that induces nonlinear excitation in a small focal area at the sample (Denk et al., 1990). In the case of two-photon excitation, the probability of emission is proportional to the square of the excitation intensity and thus decreases rapidly outside of the focal area. Thus, due to the inherent lack of out-of-focus light and use of long wavelengths, multiphoton microscopy achieves deeper optical sectioning (Centonze & White, 1998) and is less phototoxic to living samples, compared to confocal microscopy (Masters & So, 1999; Squirrell et al., 1999). Moreover, the high photon densities used for multiphoton microscopy produce second harmonic generated (SHG) light in noncentrosymmetric environments (Mohler et al., 2003; Zipfel et al., 2003a) such as are provided by collagen fibrils to allow for their detection.

There are no studies on the morphological and structural effects of chronic oxygen deprivation of bioengineered skin constructs. In this article, we use multiphoton laser scanning microscopy and SHG microscopy (MPLSM/SHG) to characterize the SM model and its response to conditions associated with skin ulceration. Prior to perturbing the SM model, we first tested the feasibility of imaging the epidermal and dermal compartments alone and in combination, and compared these images with those of native neonatal and adult human skin tissues. All three tissue types shared similar tissue architecture in both the epidermal and dermal compartments with differences being associated with the relatively greater complexity of the native tissues. Stepwise formation of the SM model revealed that both the addition of dermal fibroblasts and the presence of a stratified epithelium influence the structure of the collagen in the SM dermis. Dynamic imaging of the SM dermis allowed

quantification of collagen degradation following treatment with proteolytic enzymes. To characterize cellular changes as well as matrix changes, a hypoxia-induced tissue phenotype was reproduced and characterized in our system. From 48 to 192 h of hypoxic exposure, progressive changes not previously reported in keratinocyte morphology and collagen structure were observed. The hypoxia-induced phenotype was further examined regarding MMP-2 expression and proliferation via Ki67 staining using indirect immunofluorescence analysis. This analysis revealed changes in MMP-2 protein localization and a decrease in proliferative cells in the basal layer with hypoxic exposure. The role of MMP-2 in the SM model's response to hypoxia and subsequent reoxygenation was examined using quantitative polymerase chain reaction (PCR) analysis. Following 24 h of hypoxic exposure, MMP-2 mRNA levels initially increased and then decreased with normoxic recovery. We propose that the organotypic skin microenvironment model could ultimately be used in *in vitro* research as a model for the homeostasis and pathology of human skin including the ischemic condition.

Materials and Methods

Procurement of Human Tissues

Human neonatal foreskin tissue from a fair-skinned infant was obtained from Meriter Hospital (Madison, WI) and maintained in F12 medium at 4°C for 3 h until imaging. Human breast skin tissue from a fair-skinned adult was obtained from University of Wisconsin Hospital and was maintained in phosphate buffered saline (PBS) at 4°C for several hours until imaging. A total of three neonatal skin and four adult skin specimens were observed. Declaration of Helsinki protocols were followed.

Preparation of Human Skin Substitutes

Dermises, supplied by Stratatech Corporation (Madison, WI), consisted of polymerized type I collagen and primary human dermal fibroblast cells in 12 mm diameter tissue culture inserts (Millipore, Bedford, MA). The dermal substitute tissues were maintained at 37°C, 5% CO₂ for 3 to 19 days prior to imaging.

Skin substitutes were generated by adding NIKS keratinocytes (Allen-Hoffmann et al., 2000) or NIKS keratinocytes stably expressing green fluorescent protein (NIKS^{GFP}) (Rasmussen et al., 2010) to the dermal compartment described above. Specifically, the dermal component was allowed to contract for at least 3 days prior to seeding with keratinocytes. NIKS or NIKS^{GFP} keratinocytes were then plated at 3.5×10^5 cells per dermis. The skin substitute was maintained at the air interface without agitation using StratalifeTM media (pH 7.2–7.4) at 37°C, 5% CO₂ for at least 14 days prior to imaging. For imaging, human skin substitutes containing an epidermis and dermis, or dermis only, were removed using an 8 mm biopsy punch and placed epidermal-side down on a cover slip.

Preparation of Collagen Samples

Samples consisted of 2.0 μ m diameter fluorescent beads (365 nm exc, 415 nm em, Molecular Probes, Eugene, OR) and type I rat-tail collagen. For ungelled samples, the fluorescent beads were resuspended in acetic acid and mixed with type I collagen (3 mg/mL) at a ratio of 10% by volume. Gelled samples were prepared the same as ungelled samples, except that the fluorescent beads were resuspended in cell media and the samples were incubated at 37°C for 45 min.

Collagenase Enzyme Treatment

Bacterial collagenase from *Clostridium histolyticum* (Crescent Chemical, Hauppauge, NY) was diluted with F12 medium to a concentration of 4 mg/mL for imaging experiments or 2

mg/mL for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Aliquots of collagenase solution and control aliquots of F12 media were stored frozen until use. For each experiment, one aliquot of collagenase/F12 was thawed in an incubator for approximately 10 min. The SM dermis was removed using an 8 mm biopsy punch, placed epidermal-side down on a cover slip, and imaged. Once the image parameters were set, approximately 100 μ L of collagenase/F12 was added directly to the sample. The volume applied was enough to immerse the entire sample. In some instances, cover slips were added to prevent the sample from floating. Image stacks were acquired over time for approximately 20–40 min until the collagen had apparently degraded to its maximum extent. Data presented are representative of four separate experiments.

Extended Hypoxia Treatment

Skin substitutes were prepared as described above. At day 12 of growth, cultures were transferred to Modular Incubator Chambers (Billups-Rothenberg, Delmar, CA) and purged for a period of 10 min with a mixture of two gases including 5% carbon dioxide and a balance of nitrogen. Small reservoirs of water were added to the chambers to provide a humidified environment. Every other day nondegassed media was replenished and the chambers were purged for 10 min before being returned to the incubator. Cultures were exposed to ambient oxygen during media replenishment for approximately 5–10 min. After 48, 96, and 192 h, cultures were removed from the hypoxia chambers, removed from the insert using an 8 mm biopsy punch and placed epidermal-side down on a coverslip for imaging. Control tissues were maintained in normoxia for identical time periods. Three tissues were imaged per each time point.

Hypoxia and Reoxygenation Treatment

For reoxygenation experiments, skin substitutes were prepared and transferred to incubator chambers as described above. Cultures were sealed in purged incubator chambers for 24 h and restored to normoxic conditions for 24, 48, 72, or 96 h. Control tissues were maintained in normoxia for identical time periods.

Indirect Immunofluorescence

Primary antibodies used for indirect immunofluorescence include anti-MMP-2 mouse monoclonal antibody (IM51, Calbiochem, San Diego, CA, 1:125) and anti-Ki67 (Oncogene Research Products catalogue #NA59, currently Calbiochem, San Diego, CA, 1:20). Indirect immunofluorescence was performed on freshly cut frozen sections of paired normoxic/hypoxia-exposed NIKS^{GFP}/NIKS SM models preserved in Tissue-Tek O.C.T. (Sakura Finetek, Torrance, CA). Sections were fixed in cold acetone for 5 min and air-dried for 11–15 min. After rinsing with PBS (1 $\times$) for 5 min and blotting dry, sections were blocked with 3% normal goat serum in PBS for 30 min at room temperature in a humidified chamber. Sections were then incubated with primary antibody for 1 h at 37°C (Ki67) or room temperature (MMP-2) in a humidified chamber. After incubating with primary antibody, sections were rinsed twice with PBS and then incubated with secondary antibody Alexa 594/488 goat antimouse (Molecular Probes, Eugene, OR, 1:500) for 30 min at room temperature, in the dark. After rinsing twice with PBS, sections were stained with Hoechst 33258 (5 mg/mL) in PBS for 10 min, in the dark. Slides were then rinsed twice and stored in PBS at 4°C until use. Sections were visualized with a fluorescence microscope (Olympus, Center Valley, PA). Alexa 594, 488, and Hoechst fluorescence signals were collected individually using Texas Red, fluorescein isothiocyanate (FITC), and Hoechst optical filters, respectively, and composite images were processed using ImageJ software (Abramoff et al., 2004).

Quantification of Ki67 Positive Cells

Ki67 stained and Hoechst counter-stained fixed tissues were visualized using a fluorescence microscope (Olympus, Center Valley, PA) equipped with Texas Red and Hoechst optical filters. Six regions at a magnification of 400 $\times$ were chosen randomly based on the Hoechst signal. Total number of basal cells were manually counted based on the Hoechst signal, and the total number of Ki67 positive cells were manually counted using the Texas Red filter. Each Ki67-positive cell was verified to also contain a Hoechst signal. Ki67 staining specificity was verified with control tissues exposed to only secondary antibody. Data represent the number of Ki67-positive basal cells per region divided by the total basal cells per region averaged over six regions per tissue type.

Quantitative PCR

MMP-2 mRNA levels were measured using quantitative PCR analysis of duplicate wells from three independent experiments. Total RNA was isolated from tissues in 1 mL TRIzol Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Following DNase I treatment of 2 μ g total RNA using the TURBO DNase kit (Ambion, Austin, TX), reverse transcription was performed using oligo dT primers and M-MLV reverse transcriptase per manufacturer's instructions (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using the Chromo4 Four-Color Real-Time PCR Detector (Bio-Rad, Hercules, CA) in conjunction with SYBR Green Super-mix (Bio-Rad, Hercules, CA). The following primers were used to detect MMP-2: 5'-CGCCCATCATCAAGTTCC-3' (forward), 5'-TGTCCTTCAGCACAAACAGG-3' (reverse), and the reference gene, human cyclophilin A: 5'-CAAGGTCCCAAAGACAGCAGA A-3' (forward), 5'-CACCTGACACATAAACCTG-3' (reverse). All mRNA levels were normalized to human cyclophilin expression using the indicated primer pair. Values from reoxygenation experiments were compared to the 24 h normoxic control culture, which was arbitrarily set to 1. Opticon Monitor Software and the Genex Excel Macro (Bio-Rad, Hercules, CA) were used for data analysis.

Multiphoton Laser Scanning Microscopy

Images were recorded on a multiphoton optical workstation (OWS) at the Laboratory for Optical and Computational Instrumentation (LOCI), located at the University of Wisconsin, Madison (Wokosin et al., 2003). The components of the OWS include a laser source, a scanning head, an inverted microscope and environmental chamber, and an optical detection system. The laser source used was a femto-second Ti:sapphire laser with a pulse repetition rate of 82 MHz and a mean power of approximately 400 mW at 900 nm. The Ti:sapphire laser was pumped with a 5 W, 532 nm solid-state laser. The output power of the Ti:sapphire laser was adjusted by means of a Pockels cell attenuator. An inverted microscope (Nikon, Eclipse TE300DV) was used with a temperature controlled environmental chamber. The chamber was constructed of Lucite and enclosed the microscope stage, condenser, and objective carrier. The temperature in the vicinity of the sample was measured with a thermister probe and set to a user-specified set point. The signal detection system employed an amplifier and a Hamamatsu H7422 GaAsP photomultiplier detector.

For all imaging, an excitation wavelength of 890 nm, a 60 $\times$ water immersion lens (Nikon PlanApo NA 1.2, WD 0.5 mm), and an environmental temperature of 35 or 25 $^{\circ}$ C were used. This two-photon excitation wavelength was chosen to optimize the contrast between cells and collagen while also maximizing the penetration depth. Average power at the sample ranged from 2–12 mW and was adjusted to optimize image contrast. Image stacks were recorded using a 1024 $\times$ 1024 pixel area and an integration time of 12 μ s per pixel. Typical lateral image resolutions were 2.76 μ m per pixel, for a total scan area of 0.034 mm² (zoom 3).

The depth scanning increment was either 2 or 5 μm for all image stacks. In cases where two-photon fluorescence emission (TPFE) and SHG are collected independently, a 464 nm longpass filter (cut-on wavelength: $464 \text{ nm} \pm 3 \text{ nm}$) was used to collect TPFE, and a 445 nm custom narrow-band ($\pm 0.5 \text{ nm}$) notch filter was used to collect SHG (TFI Technologies, Inc., Greenfield, MA).

Image Analysis

Image data were acquired in WiscScan (<http://www.loci.wisc.edu/software/wiscscan>) and imported to ImageJ (Abramoff et al., 2004) and Visbio (Rueden et al., 2004) for visualization and analysis. Image intensities in each pixel within each image stack were normalized to a quadratic reference signal recorded for each image stack, to enable quantitative comparison.

Results

Nonlinear Optical Microscopy of the SM Model: Comparison with Native Tissues

To investigate the effects of oxygen deprivation and proteolytic degradation, we assessed the feasibility of nonlinear optical imaging of the dermis and epidermis of the SM model alone and in combination. MPLSM/SHG images of the SM model were compared with those of native neonatal and adult human skin tissues to assess the accuracy with which the nonlinear optical signals represent those found in native skin.

Initially, all three tissue types were compared based on traditional histological staining. Figure 1a illustrates a transverse hematoxylin and eosin (H&E) stained section of the human SM model generated with NIKS keratinocytes. The epidermal and dermal compartments are visible as well as their cellular and extracellular matrix (ECM) components. The darkly stained keratinocytes in the epidermis form stratified layers corresponding to the basal, spinous, granular, and cornified layers of stratified squamous epithelia. The enucleated squames in the upper cornified layer are partially separated from the epidermis. The darkly stained fibroblast cells are interspersed among the lightly stained collagen matrix of the dermis. For comparison, human neonatal skin (Fig. 1b) and adult skin (Fig. 1c) are also shown. Unlike the human skin substitute, the epidermaldermal interface of the native tissues is characterized by rete ridges; however, the architecture of the epidermis in both native tissues is similar to that of the SM model.

In contrast to histological analysis, MPLSM imaging describes epidermal architecture in a planar orientation. The optical contrast in the MPLSM images of Figure 1d (right) arose only from endogenous sources of TPFE and SHG. In all three tissues, all layers of the epidermis and a portion of the dermis were imaged. Overall, the epidermal compartment reflected a normal pattern of differentiation. The epidermal cells in all tissue types displayed diffuse cytoplasmic TPFE (Fig. 1d, left). In both the *in vitro* and native tissues, the cellular structures in the surface layers displayed intense nonlinear autofluorescence and took on the appearance of squames (data not shown). In the subsequent layers, the cellular structures exhibited markedly less autofluorescence and appeared large, flat, and spaced apart from each other. Cells progressively grew smaller and closer together until they appeared to be densely packed in the basal layer of the epidermis (Fig. 1j, left). In neonatal skin it is interesting to note that there is a source of contrast in the region between the cells (Fig. 1h, black arrows) that is not present in either the *in vitro* or adult skin images. Also of note are the high contrast structures present in the basal layer of adult skin (Fig. 1i, white arrows) that are not present in the *in vitro* or neonatal images. The collagen SHG signal in the dermal compartment (Fig. 1m, right) of all three tissues has a nonuniform pattern with spatially variable intensity. Comparison of MPLSM/SHG images of the SM model and

native tissues revealed that nonlinear optical signals depicting keratinocyte morphology and collagen structure are sufficiently similar to support the use of MPLSM/SHG for investigations of the SM model's response to perturbations of homeostasis.

Stepwise Formation of the SM Model Incorporating NIKS^{GFP} Keratinocytes

Although the keratinocytes utilized in the above SM model produced an adequate signal, green fluorescent protein (GFP)-expressing NIKS keratinocytes (NIKS^{GFP}) cells were explored to amplify the contrast between the cells and the dermal compartment. The stepwise formation of the SM model incorporating type I collagen embedded with human dermal fibroblasts supporting a stratified epithelium composed of NIKS^{GFP} is illustrated in Figure 2. Fluorescence signals, including GFP and cellular autofluorescence, and second harmonic signals were collected separately using 464 nm longpass (grayscale) and 490 nm shortpass (green-yellow color scale) filters, respectively, and then merged. The panels in Figure 2d exhibit a fibrillar pattern of collagen SHG that is uniform with depth without the contribution of cells. In the SM dermis (Fig. 2e), fibroblasts are visible and are present in greater numbers at the top and bottom of the sample, with some interspersed in the middle. In addition, the collagen fibril structure appears heterogeneous with regions where there is no SHG that typically, but not exclusively (arrows), correspond with the presence of fibroblasts. In Figure 2f the use of NIKS^{GFP} (grayscale) allows for improved visualization of the differentiated layers of the SM model, including cellular and subcellular features. As expected, the primary contribution to the overall signal in the epidermis is from fluorescence (grayscale) and that in the dermis is from SHG (green-yellow color scale). Because the GFP fluorescence is robust, the cellular morphology is represented in great detail and the expected pattern of differentiation is clearly displayed. The lower-cornified layer (Fig. 2f1) appears diffuse whereas subsequent layers (Fig. 2f2–6) describe an emerging cellular network. It is interesting to note a slight increase in contribution from SHG in the lower cornified layer, evidenced by a green tint in the merged images (Fig. 2f). The cells in the spinous layers (Fig. 2f2, f3) are large, flattened, and interweaved and, in contrast with corresponding data in Figure 1, exhibit a signal in the nucleus with distinct subnuclear structure. In the basal layer (Fig. 2f4) the cells are smaller, more rounded, and tightly packed. The advantage of using the NIKS^{GFP} keratinocytes to visualize the epidermal compartment of the SM model is especially apparent in the basal layer, where the endogenous cellular signal is inherently weaker compared to the cellular signal in the upper stratified layers (see Fig. 1d,g,j). At the transition between the basal layer and the dermis, basal keratinocytes and collagen fibrils are closely apposed. Beneath the basal layer, fibrillar collagen structure is less evident compared to either the SM dermis containing fibroblasts or without fibroblasts. Additionally, few fibroblasts are visible in the upper layers of the dermis. This approach provides a highly sensitive, novel readout for the differentiated state of stratified squamous epithelia and the behavior of fibroblasts within the context of an engineered skin tissue.

Quantification of Collagen Degradation in the SM Dermis

To confirm that the SHG signal in the SM model was attributed to the collagen structure of the dermis and any changes to the collagen were detectable, a collagenase treatment was performed. Figure 3a illustrates MPLSM images of the SM dermis after 4, 13, 16, and 22 min of collagenase treatment and without collagenase treatment. The SM dermis was first imaged and then treated with collagenase to perturb the collagen structure. This resulted in a dramatic decrease in signal after a time period of approximately 20 min. Fibroblast cells (arrows) served as fiduciary marks for the dermal structures, which appeared to disintegrate over time.

Figure 3b shows the average MPLSM signal per pixel as a function of time after treatment. In each case, the enzyme treated SM dermis resulted in at least an 80% decrease in signal compared to control experiments. The digestion experiments were repeated and samples analyzed by SDS-PAGE (Fig. 3c, lanes 4–7) to confirm that the decrease in SHG intensity is the result of the degradation of type I collagen. With increasing time, collagenase progressively degraded the collagen in the SM dermis to the expected lower molecular weight fragments. Taken together, these results show the feasibility of real-time imaging of the dermis and epidermis of the SM model alone and in combination.

Hypoxia-Induced Skin Tissue Phenotype

To demonstrate the utility of MPLSM/SHG imaging as a read-out of skin tissue phenotype, we chose to analyze a novel hypoxia-induced phenotype first observed by histological assessment by standard light microscopy (Strasleski et al., 2009). This phenotype is described by both H&E stained sections (Fig. 4a) and MPLSM/SHG images (Fig. 4b,c) of the NIKS^{GFP} SM model. Panels in Figure 4a juxtapose H&E stained sections of SM model tissues cultured in a low oxygen environment for 48, 96, and 192 h (right) with paired SM model tissues cultured in a normoxic environment (left). After 48 h of exposure to a hypoxic environment tissue changes are apparent. Although the overall tissue structure remains unperturbed, the keratinocytes in the lower stratified layers exhibit open spaces closely associated with the nuclear region. After 96 h of hypoxia these open perinuclear spaces are enlarged and accompanied by a disruption of tissue architecture. After 192 h of hypoxic exposure, the majority of cells in the epidermis appear dramatically enlarged and the open perinuclear spaces persist. In addition, the cells in the basal layer appear degenerated and, as illustrated in the H&E stained sections, the epidermis often separates from the dermis. The above-described tissue changes are mirrored in the MPLSM images in Figure 4b, giving the added advantage of illustrating the lateral extent of the phenotype. A comparison of the MPLSM images of normoxic and hypoxic tissues after 192 h (Fig. 4b, bottom pair) illustrates the lack of stratification and increased vertical extent of the hypoxia-induced phenotype. Panels in Figure 4c compare the morphology in the dermal compartment of the normoxic and hypoxic tissues in Figure 4b. Collagen structure in the dermis after 96 and 192 h of hypoxic exposure appears less fibrillar compared with the collagen structure in the normoxic control tissues. These results demonstrate the ability of MPLSM/SHG microscopy to identify morphological changes in the epidermis and dermis of the SM model in a low-oxygen environment.

Furthermore, visual inspection of hypoxia-exposed and normoxic control tissues showed no remarkable differences. However, upon handling, the dermal compartment of hypoxia-exposed tissues was softer and more fragile than that of the normoxic control tissues. This observation suggests that early changes in skin exposed to a low-oxygen environment may not be visually apparent.

Hypoxia-Induced Changes in MMP-2 Expression

The above results, in particular the apparent degeneration of the basal layer and the changes observed in collagen morphology with hypoxic exposure, led us to further investigate the proteolytic environment of the hypoxia-exposed tissues. The family of MMPs has been identified as a group of enzymes capable of cleaving almost every type of structural protein that makes up the ECM (Nagase et al., 2006). In general, expression occurs at a basal level during homeostasis, becoming upregulated during conditions of tissue remodeling such as are present in wound healing. It has been established by others that the type IV collagenase MMP-2 is upregulated during both normal and aberrant wound healing (Agren, 1994; Jansen et al., 2007). MMP-2 preferentially cleaves the structural components of the basement membrane: type IV collagen and laminin.

Due to its role in wound healing and its substrate specificity, we chose to investigate potential changes in MMP-2 expression with hypoxic exposure by performing indirect immunofluorescence analysis on frozen sections of normoxic control and hypoxia-exposed NIKS^{GFP} SM model tissues. Figure 5a–d describes a representative set of data illustrating MMP-2 expression (both pro and active forms) in (a) normoxic control tissues, and MMP-2 expression in tissues exposed to hypoxia for (b) 48 h, (c) 96 h, and (d) 192 h. MMP-2 expression in normoxic control tissues did not vary with time in culture (data not shown). In the normoxic control tissues, MMP-2 is prevalently expressed throughout the stratified layers of the epidermis, with slightly higher expression in the basal layer. MMP-2 is also expressed by the dermal fibroblasts in the SM model. After 96 h of exposure to hypoxia, changes in the pattern of MMP-2 expression were evident (Fig. 5c). MMP-2 expression in the upper stratified layers of the epidermis was decreased relative to that in the basal layer. After 192 h of exposure to hypoxia, MMP-2 expression in the upper layers of the epidermis was dramatically reduced, while that in the basal layer was maintained (Fig. 5d). MMP-2 expression of the dermal fibroblasts appeared to remain unaffected by hypoxic exposure. These findings confirm that MMP-2 may play a role in the morphology found in the hypoxic SM model tissues and illustrate the utility of the SM model for investigating molecular pathways.

Hypoxia-Induced Change in Proliferation

The progressive denaturation of the basal layer and changes in MMP-2 localization led us to question the self-renewal capacity of the SM model exposed to hypoxia for 192 h. To assess this we performed indirect immunofluorescence analysis on frozen sections of normoxic control and hypoxia-exposed NIKS^{GFP} SM model tissues for Ki67 expression, a marker of cellular proliferation. Of the total number of cells in the basal layer, identified by a Hoechst nuclear counter-stain, the percent Ki67-positive basal cells were enumerated and are illustrated in Figure 5e. As expected, relative to normoxic control tissues, the number of proliferating cells in the basal layer decreased with hypoxic exposure. At the most extreme time point, no basal cells were proliferating. Together, these results describe a hypoxic tissue phenotype that is characterized by degenerative changes in tissue architecture, changes in proteolytic enzyme localization, and a decrease in proliferative capacity.

Changes in MMP-2 Expression with Reoxygenation

To further investigate the proteolytic response of the SM model to hypoxia, a recovery experiment was performed. SM model tissues were exposed to 24 h of hypoxia and allowed to recover in a normoxic environment for 24, 48, 72, and 96 h. MMP-2 protein expression was assessed using indirect immunofluorescence analysis. This analysis showed that after 24 h of reoxygenation MMP-2 expression was depressed in the upper layers of the tissue relative to the normoxic control as demonstrated in Figure 6a,b. After 72 h of reoxygenation MMP-2 expression was restored in the upper layers of the tissue and was slightly greater than the normoxic control as demonstrated in Figure 6a,c. MMP-2 mRNA expression was measured using quantitative PCR and indicated the reverse trend, showing a threefold increase in expression after 24 h of recovery and decreased expression to slightly below normoxic control levels after 72 and 96 h of recovery as described in Figure 6d. These findings illustrate the capacity of the SM model to recover MMP-2 protein expression and localization upon reoxygenation.

Discussion

MPLSM optical sectioning provides both morphological and structural information in three spatial dimensions over time, unlike traditional histological staining. We present for the first time a comparison of MPLSM images of the epidermal and dermal compartments of

bioengineered, native neonatal, and adult human skin. The endogenous sources of contrast excited with 890 nm light in our MPLSM images were most likely derived from both mitochondrial flavoprotein autofluorescence and collagen SHG. A recent study discovered that cellular autofluorescence from short-term tissue cultures of cervical epithelia, excited with 488 nm (single-photon) light, colocalized with fluorescence from a probe that stains the mitochondria of living cells (Pavlova et al., 2003). This autofluorescence was attributed to mitochondrial flavin adenine dinucleotide (FAD). Other researchers used a fluorescent type I collagen specific antibody to show that the filamentous structures giving rise to a second harmonic generated signal corresponded to collagen (Zoumi et al., 2002; Brown et al., 2003). Specifically, in one study, both collagen fluorescence emission and SHG at a range of excitation wavelengths were measured. It was subsequently found that signals excited below 800 nm originate from both TPFE and SHG, and signals excited at or above 800 nm originate from SHG only. In the epidermal compartment of all three tissues, mitochondrial flavoprotein autofluorescence marks the cellular cytoplasm, which highlights the cell shape and outlines the nucleus. Cells appear small and densely packed in the basal layer, and as they progress upward forming the spinous, granular, and cornified layers, they increase in size, becoming flattened and enucleated. The upper cornified layer of all three tissues displays high contrast, which we believe is attributed to keratin autofluorescence (Smith & Melhuish, 1985; Pena et al., 2005) and/or keratin fibril SHG (Lee et al., 2009). These data confirm the expected morphological changes associated with terminal differentiation of stratified squamous epithelia.

The source of the distinctive signal at the cell-cell junctions in the lower spinous and basal layers of the neonatal skin is unknown. Given the nature of the detection method, the source localized to the cell-cell junctions is either a tissue fluorophore or a high-order structural component exhibiting SHG. We postulate that because it is not present in MPLSM images of adult skin, these structures may be involved in morphogenesis of a fully keratinizing epithelia. In MPLSM images of adult skin, there is also a high-contrast signal in the cytoplasm of a subset of cells in the basal layer that is not present in either neonatal skin or the SM model. Rajadhyaksha et al. (1995) observed a similar feature using reflectance confocal scanning laser microscopy and attributed it to light scattering of melanin in cytoplasmic melanosomes. If the melanosomes exhibit structural regularity, it is possible that they are an additional source of SHG in our images of native adult skin. Therefore, the absence of this signal is expected in our SM model because it does not contain melanocytes. In addition, we did not observe this signal in the neonatal skin sample, which perhaps contained few melanocytes.

The papillary dermis is a complex structure containing vessels and other tissue structures as well as multiple cell types within a highly organized ECM consisting of collagen, elastin, hyaluronan, and other matrix molecules. In spite of the increased complexity of the papillary dermis of native tissues compared to the SM dermis, the MPLSM/SHG images bear some similarities. In all three tissues collagen fibrillar structures are visible, and the dermal collagen appears to have undergone remodeling by the resident cells. In these images the primary source of contrast is SHG, which depends on collagen fibril formation. Differences in the SHG between the SM dermis and the dermal compartment of native tissues may be due to several factors. The SM dermis is comprised of collagen that has been chemically extracted from rat tail tendon tissue. In addition, the fibroblast concentration appears to be less than in human native dermal tissue, based on the cellularity of the dermal compartment of the tissues analyzed in our study. Regardless of these differences, the engineered dermal substrate is sufficient to support normal epithelial proliferation and differentiation.

The appearance of the collagen in MPLSM images of bioengineered skin and dermises that contained fibroblasts was found to differ from that of dermises that did not contain

fibroblasts. The collagen in the acellular dermises appeared uniform with randomly oriented fibrils that did not vary with sample depth. In the dermises with fibroblasts, the presence of cells in an image plane typically corresponded with regions where there was no SHG signal, demonstrating a lack of collagen posterior to an extended, possibly migrating cell. In restrained cultures, fibroblasts experience tension and therefore exert their own isometric mechanical forces on the matrix to migrate and reorganize the collagen fibrils in the direction of the forces (Tomasek et al., 2002; Grinnell, 2003). Moreover, in a restrained culture fibroblasts respond by taking on an activated and proliferative phenotype resulting in matrix reorganization and collagen accumulation. Conversely, in free floating cultures, fibroblasts do not experience as much tension and so do not exert their own in response. Instead they take on a resting, quiescent phenotype reminiscent of tissue homeostasis (Grinnell, 2003). Our human SM model is a primarily restrained system, which apparently induces an activated tissue phenotype involving cell migration and reorganization of the collagen matrix.

At 14 days of growth we observed that the fibroblasts in the SM dermises accumulated at the surface and at the bottom of the tissues. This phenomenon was observed in an early study by Allen and Schor in floating cultures of fibroblast-embedded collagen matrices (Allen & Schor, 1983). After two days as a restrained culture and seven additional days as floating cultures, fibroblasts embedded in collagen formed a monolayer that completely enveloped the condensed collagen matrix. This tendency of fibroblasts to migrate outward from an *in vitro* gelled collagen matrix over time occurs in both restrained and freely floating cultures. We hypothesize that the fibroblasts in the SM dermis are responding to their *in vitro* environment in part as they would in an *in vivo* wound environment, and treat the culture/air and culture/insert-bottom interfaces as wound edges. This theory is supported by studies that demonstrate cellular migration to a wound site in a skin equivalent tissue (Torkian et al., 2004). In addition, it is interesting to speculate whether the presence of a fully stratified epithelium in the composite SM model would induce a more quiescent fibroblast phenotype in the dermal compartment. A study designed to specifically address this question would need to include an optical probe associated with the fibroblast component, either by using a live cellular stain or by transgenically expressing a fluorophore in the fibroblast cells. It is also appropriate to consider the role of other ECM proteins such as elastin, fibronectin, and hyaluronan in the maintenance of a quiescent fibroblast phenotype. It has recently been shown that addition of artificially cross-linked hyaluronan inhibits collagen contraction *in vitro* in a fibroblast-embedded collagen matrix, but does not alter the fibroblast morphology, as measured by F-actin immunofluorescence staining (Mehra et al., 2006).

Optical contrast in the epidermis of the SM model was dramatically improved through the use of NIKS^{GFP} cells. This improvement enhances the ability to visualize perturbations of cell morphology in this model. When color-merged with the SHG-filtered data, there appeared to be a source of SHG occurring in the upper layers of the epidermis. Known cellular sources of SHG include intermediate filaments such as actin of the cytoskeleton and actomyosin of skeletal muscle (Campagnola & Loew, 2003). It is well known that during the process of terminal differentiation in keratinizing epithelia such as skin, keratin filaments are bundled and processed to form the primary structural element of the outermost cornified layer. Given these facts, we postulate that the source of SHG in the upper epidermal layers of the SM model is nascent keratin fibrils. This could be tested by colocalizing a keratin-specific antibody with the SHG signal.

We showed the feasibility of monitoring collagen remodeling in the human SM model by inducing collagen degradation in a dermal substitute tissue with bacterial collagenase and observing the changes that occurred over time. The precipitous decrease in the collagen

SHG signal we observed was confirmed to be due to collagen degradation using SDS-PAGE analysis. Our results support the findings of Brown and coworkers (2003), who observed collagen degradation *in vivo* in a tumor model system consisting of an Mu89 melanoma implanted in the dorsal skinfold chamber of immunodeficient mice, with collagenase and relaxin treatment. They demonstrated that SHG allows dynamic imaging of collagen modification in an *in vivo* tumor model, and also that a pharmacologic agent such as relaxin increases the diffusivity of tumor tissue, potentially improving drug delivery to tumors.

Having demonstrated that MPLSM/SHG is a sufficiently sensitive technique to visualize morphological and structural changes in the SM model, we confirmed this by observing tissue changes with hypoxic exposure. Both traditional histology and MPLSM/SHG microscopy illustrate progressive changes beginning in the basal layer and ultimately affecting the entire epidermal compartment. The cellular morphology after 192 h of hypoxic exposure can be described as a ballooning degeneration characterized by cellular enlargement and cytoplasmic vacuolation. MPLSM/SHG microscopy provides additional information on the lateral extent of the hypoxia-induced phenotype in a living tissue. In addition, changes in dermal collagen fibrillar structure were apparent after 96 h of hypoxic exposure. These cellular and extracellular tissue changes were not perceptible upon visual inspection of the tissues, indicating that MPLSM/SHG imaging is a potentially useful diagnostic tool for identification of conditions that may lead to the development of ulceration.

Following hypoxic exposure the ballooning phenotype of keratinocytes progressed upward to suprabasal cells in the epidermis while MMP-2 protein expression was attenuated, ultimately being maintained in the basal layer only. While the MMP-2 antibody used in this study does not distinguish between pro and active forms, if the basal expression at 192 h indicates active MMP-2, it is possible that this activity contributed to the erosion of the basement membrane, resulting in a separation of the epidermal and dermal compartments as evidenced by the histological analysis. This is supported by a related study that showed that hypoxic exposure (1% O₂, 48 h) attenuated MMP-2 activation and invasion of human cardiac myofibroblasts (Riches et al., 2009).

The cells in the basal layer of tissues exposed to hypoxia for 192 h were proliferation deficient, suggesting the tissue had entered a quiescent state with an attenuated ability to self-renew. This response to chronic oxygen deprivation may explain in part the inability of chronic wounds to reepithelialize. Further studies are needed to confirm the establishment of a quiescent state, including measures of cell viability after 192 h of hypoxia.

The hypoxia-induced decrease in MMP-2 protein levels was reversed upon reoxygenation following 24 h of hypoxic exposure. This result together with evidence of proliferating basal cells after 48 h of hypoxic exposure strongly supports the presence of a viable tissue containing proliferating keratinocytes after a 24 h hypoxic insult. Interestingly, MMP-2 protein levels after 96 h of normoxic recovery are increased above that of the normoxic control. This observation is further supported by an inverse trend in MMP-2 mRNA expression levels. These data are suggestive of a dysregulation in MMP-2 activity upon reoxygenation that may mimic in part proteolytic imbalances that occur during ischemic-reperfusion injury. Furthermore, these findings support the use of the SM model to investigate the relationship between duration of hypoxic insult and reversibility of the hypoxic phenotype.

The ability of MPLSM/SHG microscopy to characterize the hypoxia-induced phenotype and proteolytic collagen degradation in a living skin equivalent tissue illustrates the value of this technique for studying aberrant wound healing. In contrast to deficient healing of a chronic

wound, excessive healing may occur in response to surgical trauma that involves overproduction of ECM proteins and manifests in fibrosis or formation of contractures (Diegelmann & Evans, 2004). Torkian et al. (2004) applied MPLSM/SHG microscopy to the study of fibrotic response to surgical incisional wounding in bioengineered constructs of human skin incorporating normal or keloid-derived fibroblasts. Yeh et al. (2004) used similarly engineered skin constructs to illustrate the consequences of laser thermal injury using both optical coherence tomography and MPLSM/SHG microscopy. These studies illustrate the use of MPLSM/SHG microscopy to identify increased collagen density in response to incisional wounding and collagen denaturation due to thermal injury, respectively. In addition, changes in cellular phenotype and migration were observed at the wound sites. Raub et al. (2007) recently reported that collagen TPFE and SHG signals can be used in combination to assess the overall mechanical strength of collagenous tissues directly, indicating that subtle changes in tissue integrity can be measured. While new modalities, such as MPLSM/SHG microscopy, that provide detailed characterization of tissue response to wounding, the wound itself, and the response to therapy, are important in treatment, these same modalities can also be applied toward understanding the underlying pathophysiological factors that lead to aberrant wound healing.

The data presented in this article suggest that conditions that are physiologically relevant to ischemia such as chronic oxygen deprivation and increased proteolytic activity cause detectable changes in our experimental model system. Therefore the SM model has potential for further development as a model of ischemic skin. Ideally the system would model the full disease progression from onset to chronic ischemia, and ultimately to the development of ulceration thereby providing a tool for the testing of preventative and therapeutic interventions. As pointed out by Dalton et al. (2007), we first need a better definition of the preulcer state. Correlative evidence in the literature suggests that prior to injury, ischemic skin is characterized at least in part by increased lactate, TGF- β , VEGF, angiogenesis, chronic oxygen deprivation, and increased, yet incomplete collagen synthesis and remodeling resulting in increased susceptibility to proteolytic degradation (Dalton et al., 2007). In addition to environmental perturbations such as chronic oxygen deprivation, the SM model is amenable to chemical, biological, and genetic perturbations, for example, by deprivation of nutrients, the introduction of immune cells, and by transgenic expression of genes that either suppress or enhance the disease state, respectively. In summary, the SM model provides an important, innovative tool for the *in vitro* analysis of phenotypic data as well as the corresponding molecular mechanisms that dictate regulation and deregulation in epithelial tissue.

Summary

In this study we have analyzed, for the first time, the effects of chronic oxygen deprivation on a bioengineered model of human interfollicular epidermis. Histological analysis and MPLSM/SHG imaging revealed a progressively degenerating ballooning phenotype of the keratinocytes that manifested after 48 h of hypoxic exposure. MPLSM/SHG images of the dermal compartment revealed a decrease in collagen structural order with hypoxia, indicating a decrease in tissue integrity. Immunofluorescence for MMP-2 illustrated an overall decrease in MMP-2 protein expression in the epidermis with a shift in spatial localization to the basal layer, potentially explaining the weakening of the dermal-epidermal interface. Immunofluorescence for Ki67 revealed a total lack of proliferation in the basal cells after 192 h of hypoxia, indicating that the tissue had entered a quiescent state, temporarily losing its ability to self-renew. Reoxygenation after 24 h of hypoxia suggested a dysregulation of MMP-2 protein and mRNA levels upon normoxic recovery. We propose that these conditions would provide a significant disadvantage to skin in producing a normal

wound healing response and, in part, validate Dalton et al.'s (2005, 2007) proposed mechanism for the development of chronically ischemic skin.

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Figure 1.

Histological sections and MPLSM images of the SM model and native skin. Five μm thick H&E stained sections of (a) the SM model at 14 days, (b) neonatal human skin, and (c) adult human skin. Note relatively darker staining in the epidermis compared to the dermis. Blood vessels are present in the neonatal skin and appear as bright pink-stained structures. Scale bars are 50 μm . TPFE + SHG images of the (d,g,j,m,p) SM model, (e,h,k,n,q) neonatal foreskin, and (f,i,l,o,r) adult human skin. Scale bar is 20 μm . Panels representing the SM model describe image planes in the (d,g,j) epidermis and (m,p) dermis and are located, from top to bottom, 26, 50, 76, 84, and 110 μm below the tissue surface. Panels representing neonatal skin describe image planes in the (e,h,k) epidermis and (n,q) dermis and are located, starting with panel h, 20, 30, 44, and 58 μm below the depth of panel e. Panels representing adult human skin describe image planes in the (f,i,l) epidermis and (o,r) dermis of adult skin and are located, from top to bottom 32, 42, 50, 66, and 76 μm below the tissue surface. All MPLSM images were collected without the use of optical filters for this experiment. Each frame is contrast enhanced for clarity, so image intensities are not comparable.

Figure 2.

Stepwise formation of the SM model. Illustrations representing the SM dermis (**a**) without fibroblasts, (**b**) with fibroblasts, and (**c**) the SM model. TPFE + SHG images of (**d**) a SM dermis without fibroblasts, (**e**) a SM dermis with fibroblasts, and (**f**) the SM model. Arrows in **e** indicate regions void of SHG signal that are not associated with dermal fibroblasts. TPFE and SHG signals were collected separately using 464 nm longpass and 490 nm shortpass filters, respectively. The 464 nm-filtered and 490 nm-filtered signals were attributed a grayscale and green-yellow color scale, respectively, and merged. Each image stack describes the full thickness of the respective tissue: (**d**) 30 μm , (**e**) 60 μm , and (**f**) 104 μm . Axial distances between adjacent panels are (**d**) 6 μm , (**e**) 12 μm , (**f**, top 4 panels) 20 μm , and (**f4–f6**) 8 μm . Panels **d1** and **e1** represent the surface of each respective tissue, while panel **f1** represents the image plane 26 μm below the tissue surface. Scale bar is 50 μm . Each frame is contrast enhanced for clarity, so image intensities are not comparable.

Figure 3.

Enzymatic degradation of the SM dermis. **a:** TPFE + SHG images of the SM dermis after 4, 13, 16, and 22 min of collagenase treatment, and without collagenase treatment. All images were recorded at the same depth (10 μm). Fibroblast cells are indicated by arrows. Note slight fibroblast drift as collagen degradation progresses. All image intensities are comparable. Scale bar is 20 μm . **b:** Normalized image intensity over time for collagenase treated (black) and control (striped) SM dermises of **a**. These data are representative of four separate experiments. **c:** SDS-PAGE of: lane 1, bacterial collagenase (2 mg/mL); lane 2, ungelled rat tail tendon collagen; lane 3, SM dermis; lanes 4–7, SM dermis after 5, 10, 15, and 20 min of collagenase digestion.

Figure 4.

Hypoxia-induced skin tissue phenotype. Five μm thick H&E stained sections of SM model tissues cultured in a normoxic environment for (a, left) 48, 96, and 192 h and (a, right) a hypoxic environment. Scale bar is 50 μm . (b,c) Selected TPFE + SHG image planes of SM model tissues grown in parallel with the tissues in a. The location of the image planes in b are indicated in a by red arrow heads and occur (b, top two panels) 12 μm and (b, bottom four panels) 48 μm above the epidermal-dermal interface. The location of the image planes in c occur 6 μm beneath the epidermal-dermal interface. Scale bar is 50 μm . All MPLSM images were collected without the use of optical filters for this experiment.

Figure 5. Hypoxia-induced change in MMP-2 expression and proliferation. Indirect immunofluorescence illustrating MMP-2 expression in a normoxic control tissue at (a) 96 h, and MMP-2 expression in tissues exposed to hypoxia for (b) 48 h, (c) 96 h, and (d) 192 h. Epifluorescence from Alexa Fluor 594 and Hoechst dyes were collected individually using Texas Red and Hoechst optical filters, respectively. Scale bar is 50 μ m. e: Percent Ki67-positive basal cells in normoxic- and hypoxia-exposed tissues. Error bars are mean $\pm$ SD.

Figure 6.

Change in MMP-2 protein and mRNA expression during tissue reoxygenation. Indirect immunofluorescence illustrating MMP-2 expression in (a) a normoxic control tissue, and after (b) 24 h and (c) 72 h of normoxic recovery following 24 h of hypoxic exposure. Epifluorescence from Alexa Fluor 488 and Hoechst dyes were collected individually using FITC and Hoechst optical filters, respectively. Scale bar is 50 μ m. d: MMP-2 mRNA expression level during reoxygenation following 24 h of hypoxic exposure, relative to MMP-2 mRNA levels of a normoxic control tissue. Data presented are representative of three independent experiments, and error bars in d are mean of duplicate samples $\pm$ SD.