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Spatiotemporal progression of cell death in the zone of ischemia surrounding burns

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Abstract

Burns are dynamic injuries, characterized by progressive death of surrounding tissue over time. Though central to an understanding of burn injury progression, the spatiotemporal degrees and rates of cellular necrosis and apoptosis in the zone of ischemia surrounding burns are not well characterized. Using a validated porcine hot comb model, we probed periburn tissue at 1, 4, and 24 hours after injury for high mobility group box 1 (HMGB1) as a marker of necrosis and activated cleaved caspase 3 (CC3a) as a marker of apoptosis, followed by spatiotemporal morphometric analysis. We found that necrosis was the most prominent mechanism of cell death in burn injury progression, with significant progression between 1 and 4 hours post-burn. Apoptosis appeared not to play a role in early burn injury progression, but was observed in cells at the interface of necrotic and viable tissue at 24 hours post-burn. Our findings imply that intervention within the first 4 hours following injury is likely necessary to limit burn injury progression. Additionally, based on HMGB1 staining patterns, we define distinct early, intermediate and late pathological signs of cell necrosis that may facilitate delineation of causal mechanistic relationships of burn injury progression in vivo.

Keywords

burn; injury progression; necrosis; apoptosis; HMGB1; caspase 3a; animal models; immunohistochemistry

Introduction

Burns are dynamic injuries, characterized by an irreversible central zone of coagulation necrosis surrounded by a zone of ischemia that, while initially viable, may ultimately succumb to progressive injury resulting in tissue death over the subsequent 24–72 hours.¹ This phenomenon is of particular clinical relevance, for it can result in the conversion of a partial-thickness burn, which will heal spontaneously, to a full-thickness burn, in which the loss of adnexal elements such as hair follicles necessitates skin grafting or more involved surgical management to achieve wound closure. Additionally, burn injury progression has been associated with worse scarring, greater wound contracture, and an increased likelihood

of wound infection.² Thus, potential interventions to limit burn injury progression promise to significantly reduce morbidity in patients suffering partial-thickness burns.

Elucidation of the relative roles of cellular apoptosis and necrosis in progression of the zone of ischemia from viable to non-viable is central to an understanding of burn injury progression. Despite its importance, the differential spatiotemporal degrees and rates of necrosis and apoptosis in burn injury progression are not well characterized.³⁻⁵ Such knowledge will both inform efforts to delineate the molecular mechanism of burn injury progression and provide objective endpoints by which to track the evolution of burn wounds and to evaluate potential therapeutic interventions in both experimental models and patients. In the present study we employed immunohistochemistry to characterize these two mechanisms of cell death surrounding burn wounds in a porcine model at graduated distances and various time points after initial injury.

As an *in vivo* marker of cell necrosis, we used translocation of high mobility group box 1 (HMGB1) from nucleus to cytoplasm and extracellular space. HMGB1 is a highly conserved chromatin-bound protein expressed in most eukaryotic cells that facilitates DNA binding by a diversity of transcription factors and has been shown to function as an alarmin, activating the innate immune system and inflammatory pathways upon release to the extracellular environment.⁶⁻⁸ Cells contain on average 10⁶ copies of HMGB1 per nucleus.⁹ It has been shown that upon cell death by necrosis HUVEC and HeLa cells passively release HMGB1 into the extracellular space as the cell membrane disintegrates and that remnants of necrotic cells are no longer associated with HMGB1.⁹ Once released to the extracellular space, HMGB1 serves as a signal of traumatic cell death, mediating a number of inflammatory processes through high-affinity binding of the receptor for advanced glycation end-products (RAGE) on target cells.⁸ HMGB1 has been shown to activate human microvascular endothelial cells *in vitro*, resulting in endothelial upregulation of leukocyte adhesion molecules ICAM-1 and VCAM-1 as well as the release of TNF α , IL-8, and MCP-1.¹⁰ HMGB1 has also been shown to play a role in wound repair as a chemokine for vascular smooth muscle cell, fibroblast, and keratinocyte migration and as a proangiogenic cytokine.^{8,9,12} In addition to passive release from necrotic cells, HMGB1 can also be actively secreted by activated macrophages and monocytes, which may represent an evolutionary adaptation of the necrotic alarmin function of HMGB1 to enable upregulation of inflammatory and repair pathways in the absence of actual cell death.¹⁰ Secretion involves the acetylation of lysine residues on HMGB1 to induce its release from chromatin and translocation via a nuclear export signal to the cytoplasm where it is concentrated in secretory lysosomes that can undergo exocytosis.¹³ HMGB1 lacks a signal peptide for targeting to the endoplasmic reticulum and Golgi apparatus, and is thus never secreted through this pathway.¹³

Application of HMGB1 to the study of burn injury progression requires an understanding of the expression of HMGB1 in cellular elements of the skin. Immunohistochemical studies have shown that nuclei of human arterial endothelial cells contain high levels of HMGB1, while HMGB1 was not detected in human arterial smooth muscle cells.⁹ The latter finding is consistent with *in vitro* experiments showing very low levels of HMGB1 expression by rat smooth muscle cells.⁹ *In vitro* studies have demonstrated that HMGB1 is located primarily in the nucleus of mouse fibroblasts, is not released from unstressed fibroblasts, and that induction of necrosis by addition of H₂O₂ results in HMGB1 release to the extracellular medium.¹⁵ Furthermore, it has been shown *in vitro* that in mouse fibroblasts treated with trichostatin A (TSA), a histone deacetylase inhibitor, HMGB1 translocates to the cytoplasm but is not packaged into secretory vesicles.¹⁵ This may indicate that like endothelial cells, intact fibroblasts do not actively secrete HMGB1. Cytoplasmic translocation of HMGB1 has been demonstrated *in vitro* in fibroblasts stressed with TNF α , though HMGB1 was not

detected in the extracellular medium in these experiments.¹² Finally, immunohistochemical studies have shown that HMGB1 is located in the nucleus of viable human epidermal cells and is found in the cytoplasm of epidermal cells adjacent to non-healing ulcers in both diabetic and non-diabetic skin.¹²

We chose HMGB1 because it is an ideal marker to differentiate cellular necrosis and apoptosis. In contrast to the passive spilling of HMGB1 by necrotic cells, apoptotic cells do not release HMGB1.¹⁴ During apoptosis, histone deacetylation results in an increased affinity of HMGB1 for chromatin and thus tight binding of HMGB1 to DNA of apoptotic cells as it condenses and is orderly packaged into apoptotic bodies for phagocyte clearance.¹⁴ Given the role of HMGB1 as an important inflammatory mediator, its retention during apoptosis, a non-inflammatory process, is not surprising.

As an *in vivo* marker of cell apoptosis, we used cleaved caspase 3a (CC3a). Apoptosis is a highly regulated, non-inflammatory mode of cell death in which cellular components are orderly dissembled and packaged into apoptotic bodies to be cleared by phagocytes. Apoptosis can be a physiological process, as in development, or a pathological process in response to stress signals such as TNF α . In either case, apoptosis is executed by a series of zymogens, known as caspases, which undergo successive activation in an orderly proteolytic cascade. This cascade is initiated by death signals such as TNF α and culminates with cleavage and activation of caspase 3 to form cleaved caspase 3 (CC3a), which then proceeds to degrade cellular components. Immunohistochemical detection of CC3a thus indicates a cell that is fully committed to apoptotic cell death.¹⁶

Few studies have investigated the relative degrees of cellular necrosis and apoptosis in burn injury progression. Prior immunohistochemical studies have relied on subjective scoring of changes in cellular architecture to determine burn depth and progression.¹⁷ Immunohistochemical staining for vimentin has been used to determine burn depth, but does not correlate with cell death during early 1 and 4 hr time periods and does not provide information regarding the mechanism of cellular death.¹⁸ To our knowledge our group was the first to use HMGB1 as an *in vivo* marker of necrosis in skin. We employed immunohistochemical detection of HMGB1 and CC3a to probe for evidence of necrosis and apoptosis, respectively, of various dermal elements in a murine hot comb burn model.¹⁹ Only one subsequent study has employed HMGB1 *in vivo* to assess necrosis in burn wounds.⁵ The present study represents a substantial expansion of our earlier work, and to our knowledge, represents the first study to apply morphometric analysis of the spatiotemporal degrees of necrosis and apoptosis in the zone of ischemia of burn wounds.

Methods

Institutional Approval

Our study protocol was conducted with full approval of the Institutional Review Board (COHRIS) and the Division of Laboratory Animal Research (DLAR) of Stony Brook University Medical Center.

Experimental Model

A previously validated porcine hot-comb burn model was employed.²⁰ Burns were created using a 150 g brass comb (Fig. 1A) with four 10 $\times$ 25 mm prongs separated by 5 mm interspaces. The comb was allowed to equilibrate for 5 minutes in a 100°C water bath, blotted dry, and applied to the dorsum of a 25 kg domestic pig for 30 seconds. Application of the comb created four full-thickness burns under the prongs that were separated by unburned interspaces, which represent the zones of ischemia in our model (Fig. 1B). Eight evenly spaced comb burns were created on the dorsum of two experimental animals.

Three series of 8 mm punch biopsies were taken at 1, 4, and 24 hours post-burn from different burn wound interspaces from comb burns on two untreated pigs. Each 8 mm biopsy encompassed the 5 mm unburned interspace with 1.5 mm of burned tissue on either side (Fig. 1B). For each specimen, a single burn was chosen for analysis with its corresponding zone of ischemia (Fig. 1C). An 8 mm biopsy from unburned porcine skin served as a control.

Tissue Processing

Biopsies were immediately fixed in formalin. Specimens were subsequently paraffin-embedded and cut into 5 μ m sections. The procedure for unmasking of antigens was antigen dependent, as detailed below. Specimens were then probed with anti-high mobility group box 1 antibodies (HMGB1; Abcam, Cambridge, MA) for evidence of necrosis and anti-cleaved caspase 3a antibodies (CC3a; Cell Signaling, Beverly, MA) for evidence of apoptosis. Adjacent sections were probed with anti-CD31 antibodies (R&D Systems, Minneapolis MN) to identify endothelial cells, and identification of blood vessels was facilitated by anti-laminin staining (Abcam, Cambridge, MA) to delineate basement membranes.

HMGB1 Staining

After deparaffinization and rehydration, antigen unmasking was accomplished by heating slides in an oven for 3 hours followed by incubation in Trilogy antigen retrieval solution (Cell Marque, Rocklin CA) for 45 minutes at 90°C. Slides were allowed to cool for 30 minutes at room temperature, washed with tris-buffered saline (TBS) and then incubated with anti-high mobility group box 1 antibodies (HMGB1; Abcam, Cambridge, MA) at a concentration of 1:200 in TBS at room temperature for 1 hour. Slides were again washed with TBS and detection of primary antibodies was performed using the MACH4 Universal AP Polymer Kit (Biocare Medical, Concord, CA), a biotin-free detection system in which a probe directed against primary mouse antibodies is followed by an alkaline phosphatase polymer that binds the probe. Slides were incubated with the MACH 4 Mouse Probe for 20 minutes at room temperature, washed with TBS, and then incubated in MACH 4 MR AP-Polymer for 20 minutes. Slides were again washed with TBS and then incubated in Vulcan Fast Red (red chromogen) for 20 minutes at room temperature. A light hematoxylin counter stain (2 dips) was applied for visualization of non-necrotic nuclei. Finally, slides were rinsed in distilled water, dehydrated, cleared, and mounted for analysis.

CC3a Staining

After deparaffinization and rehydration, antigen unmasking was accomplished by incubating slides in Trilogy antigen retrieval solution (Cell Marque, Rocklin CA) for 1 hour at 92°C. Slides were allowed to cool for 30 minutes at room temperature and then incubated in Peroxidized solution (Biocare Medical, Concord CA) for 5 minutes at room temperature to quench endogenous peroxidases. Slides were washed with phosphate buffered saline (PBS) and then blocked for 30 minutes in normal goat serum (Vector Labs, Burlingame CA). Slides were again washed with PBS and then incubated at 4°C overnight with anti-cleaved caspase 3a antibodies (CC3a; Cell Signaling, Beverly, MA) at a concentration of 1:100 in block solution. Following overnight incubation, slides were washed in PBS and incubated for 30 minutes at room temperature with biotinylated goat anti-rabbit antibodies at a concentration of 1:500 in block solution. Slides were again washed in PBS and incubated with avidin-biotin-peroxidase complex (ABC) reagent (Vectastain ABC kit, Vector Labs Inc., Burlingame, CA) for 30 minutes at room temperature. The slides were then washed in PBS and incubated in 3,3-diaminobenzidine (brown chromogen) for 6 minutes at room temperature. A light hematoxylin counter stain (2 dips) was applied for visualization of non-

apoptotic nuclei. Finally, slides were rinsed in distilled water, dehydrated, cleared, and mounted for analysis.

CD31 Staining

After deparaffinization and rehydration, antigen unmasking was accomplished by incubating slides in Trilogy antigen retrieval solution (Cell Marque, Rocklin CA) for 2 hours at 82°C. Slides were allowed to cool for 30 minutes at room temperature, washed in TBS and Tween (Thermo Scientific, Fremont CA), and then incubated with anti-CD31 antibodies (R&D Systems, Minneapolis MN) at a concentration of 1:50 in TBS at 4°C for 16 hours. Slides were then washed with TBS and detection of primary antibodies was performed by incubation with monoclonal anti-goat/sheep IgG (Sigma-Aldrich, St. Louis MS) at a concentration of 1:50 at room temperature for 1 hour. Slides were again washed with TBS and then incubated in Vulcan Fast Red (red chromogen) for 10 minutes at room temperature. A light hematoxylin counter stain (2 dips) was applied for visualization of cell nuclei. Finally, slides were rinsed in distilled water, dehydrated, cleared, and mounted for analysis.

Laminin Staining

After deparaffinization and rehydration, antigen unmasking was accomplished by incubating slides in Trilogy antigen retrieval solution (Cell Marque, Rocklin CA) for 2 hours at 82°C. Slides were allowed to cool for 30 minutes at room temperature, washed in TBS and Tween (Thermo Scientific, Fremont CA), and incubated in protein block for 10 minutes (Biocare Medical, Concord CA). Slides were washed in TBS for 5 minutes and then incubated with anti-laminin antibodies (Abcam, Cambridge, MA) at a concentration of 1:25 in TBS at 4°C for 16 hours. Slides were again washed with TBS and detection of primary antibodies was performed using the MACH4 Universal AP Polymer Kit (Biocare Medical, Concord, CA). Slides were incubated with the MACH 4 Mouse Probe for 20 minutes at room temperature, washed with TBS, and then incubated in MACH 4 MR AP-Polymer for 30 minutes. Slides were again washed with TBS and then incubated in Vulcan Fast Red (red chromogen) for 20 minutes at room temperature. A light hematoxylin counter stain (2 dips) was applied for visualization of cell nuclei. Finally, slides were rinsed in distilled water, dehydrated, cleared, and mounted for analysis.

Assessment of Necrosis and Apoptosis

Viable cells are characterized by HMGB1 nuclear staining with no or minimal cytoplasmic staining (Figures 2 and 4D, respectively). Necrotic cells were identified by loss of nuclear HMGB1 staining.^{8,9} It is important to note that chromatin appears black when nuclei stain with both hematoxylin and HMGB1. In contrast, chromatin appears blue when nuclei do not stain with HMGB1, i.e. stain with hematoxylin only. Apoptotic cells were identified by cytoplasmic staining with CC3a.¹⁶ Morphometric measurements were made using a calibrated microscope eyepiece reticle and grid.

Quantification of Vascular Endothelial Cell Necrosis

Using a calibrated eyepiece reticle and grid at 20× power, the burn interspace of each specimen was divided into eight zones beginning at the burn edge and moving horizontally to mid-interspace (Fig. 1C). Each zone measured 0.25 mm wide by 1 mm deep with respect to the epidermal basement membrane. All vessels present in each zone were noted based on laminin staining patterns (Fig. 3A). All endothelial cells were then identified based on CD31 staining and cell morphology (Fig. 3B). Next, adjacent sections stained for HMGB1 were used to quantify the total number of endothelial cells present in each zone. The proportion of endothelial cells without nuclear HMGB1 staining, indicative of cell necrosis, was noted

(Fig. 3C). Counts were performed by a single observer for all three specimens at each time point.

Statistical Analysis

For each of the zones described above, percent endothelial cell necrosis was calculated by dividing the total number of necrotic endothelial cells by the total number of endothelial cells present. All zones were then compared for differences in percent endothelial cell necrosis using a single repeated measures analysis of variance analysis (AVOVA). Independent variables in the analysis were time (1, 4, or 24 hours) and zone (i.e. proximity to the burn), while the dependent variable was percent endothelial cell necrosis. Statistical analysis was performed using SPSS Version 18.0 for Windows (SPSS, Inc). Two-tailed P-values were used for all comparisons and an alpha level of 0.05 was used as a threshold for statistical significance.

Results

Necrosis

Burned Tissue Below the Hot Comb—Complete tissue necrosis beneath the hot comb burn sites was observed at all time points—1, 4, and 24 hours post-burn—as indicated by both morphological changes and HMGB1 staining. Burned epidermis at all 3 time points underwent complete coagulation necrosis as evidenced by loss of keratinocyte nuclear HMGB1 staining and architectural distortion, with nuclear streaming, or “string bean nuclei,” secondary to direct heat transfer (Figs. 4A and 5A, F, K). A small degree of epidermal extracellular HMGB1 staining was observed at 1 hour, which was decreased at 4 hours and absent by 24 hours. Two areas of HMGB1 staining in the epidermis at 24 hours (Fig. 5K) likely represent artifacts of tissue processing.

Burned dermis at all 3 time points was characterized by interstitial cell (fibroblast, tissue macrophage, dendritic cell) and vascular endothelial cell pyknosis, karyolysis, and karyorrhexis, as well as disruption of the interwoven, fibrillar collagen network. Cellular number in burned dermis appeared reduced compared to control tissue. Interstitial cells and vascular endothelial cells showed a complete absence of HMGB1 staining indicative of cellular necrosis. Subtle differences in extracellular HMGB1 staining were observed at 1, 4, and 24 hours. At 1 hour, prominent extracellular HMGB1 staining was observed (Fig. 5A), while at 4 hours, extracellular HMGB1 staining is markedly reduced and is concentrated in the perivascular space (Fig. 5F). By 24 hours, there is a complete absence of extracellular HMGB1 staining (Fig. 5K). This time-dependent decrease in extracellular HMGB1 in the burned dermis may be a result of HMGB1 proteolysis or diffusion away from areas of cell necrosis.

Zone of Ischemia Lateral to the Hot Comb

Epidermal Progression: At 1 hour post-burn, keratinocytes adjacent to the burn displayed a loss of nuclear HMGB1 staining indicative of cell necrosis extending to 0.75 mm from the burn, albeit HMGB1 staining patterns varied by proximity to the burn (Fig. 4). The HMGB1 staining pattern transitioned from absence beneath the hot comb (Fig. 4A), to diffuse extracellular staining from the burn edge to 0.4 mm from the burn (Fig. 4B), to concentrated extracellular and cytoplasmic staining from 0.4 mm to 0.75 mm from the burn (Fig. 4C), to nuclear staining indicative of cell viability from 0.75 mm to 2.0 mm from the burn (Fig. 4D). The area of concentrated extracellular and cytoplasmic staining (Fig. 4C) represented a “conversion zone” between necrotic and viable keratinocytes. These staining patterns were mirrored by a gradient of decreasing architectural distortion moving away from the burn. Keratinocyte architecture gradually transitioned from nuclear streaming at the burn edge

(Fig. 4B) to round, pyknotic, angular nuclei between 0.5 and 0.75 mm from the burn (Fig. 4C, transition shown at lower power in Fig. 5B-C). Further from the burn, epidermal architecture was intact (Figs. 4D and 5C-E).

Over time, the pattern of diffuse extracellular HMGB1 staining (Fig. 4B) extended further from the burn and was accompanied by a progression of the transition point between necrotic and viable keratinocytes (compare Fig. 5A-E, F-J, K-O). At 4 hours post-burn, epidermal HMGB1 staining was similar to the pattern observed at 1 hour, but shifted an additional 0.45 mm from the burn (Fig. 5F-J). This was again mirrored by a shifted gradient of keratinocyte architectural changes. The conversion zone between necrotic and viable keratinocytes was observed from 0.9 to 1.2 mm lateral to the burn (Fig. 5H-I). This represents a 0.5 mm progression of this epidermal transition zone from 1 hour (Fig. 5B-C). From 1.2 to 2.0 mm from the burn, epithelial architecture was intact and nuclear HMGB1 staining indicated keratinocyte viability (Fig. 5I-J). Increased extracellular HMGB1 staining of this area compared to control tissue may represent diffusion of HMGB1 released from adjacent necrotic cells.

By 24 hours post-burn, epidermal necrosis progressed to 1.8 mm from the burn, encompassing nearly the entire zone of ischemia (Fig. 5L-O). HMGB1 staining displayed an additional 0.6 mm shift of the epidermal conversion zone between necrotic and viable keratinocytes compared to 4 hours post-burn (compare Fig. 5I and O). Thus, a time-dependent, moving gradient of HMGB1 staining and architectural changes was evidenced by a horizontal progression of epidermal necrosis into the zone of ischemia over time. Interstitial cells and vascular endothelial cells also displayed a similar spatiotemporal variation in HMGB1 staining patterns, as detailed below.

Dermal Progression: At each time point, vascular endothelial cell necrosis, as determined by presence or absence of nuclear HMGB1 staining, varied by proximity to the burn edge (Fig. 6). At 1 hour post-burn, 75% of vascular endothelial cells were found to be necrotic as far as 0.75 mm from the burn. Only 45% vascular endothelial cell necrosis was observed in the 0.75-1.0 mm zone and an even smaller percentage of vascular endothelial cells were necrotic between 1.0 and 1.25 mm from the burn. In contrast, a great majority of endothelial cells further than 1.25 mm from the burn appeared viable at 1 hour post-burn. Interstitial cells necrosis, as evidenced by a loss of nuclear HMGB1 staining and architectural disruption, was observed up to 0.75 mm from the burn (Fig. 6A-B), while HMGB1 staining of interstitial cells beyond this point indicated cell viability (Fig. 6C). A moderate degree of perivascular HMGB1 staining was observed at 0.25 mm away from the burn (Fig. 6A), with increased perivascular staining evident in necrotic areas further from the burn (Fig. 6B). No extracellular HMGB1 staining was observed further than 1.25 mm from the burn (Fig. 6C), where all vascular endothelial and interstitial cells remained viable at the 1-hour time point.

A significant progression of vascular endothelial cell necrosis occurred between 1 and 4 hours post-burn. Whereas the majority of vascular endothelial cells further than 0.75 mm were viable at 1 hour, by 4 hours nearly 100% of vascular endothelial cells were necrotic as far as 1.5 mm from the burn. A large decrease in percent vascular endothelial cell necrosis to 49.3% occurred between 1.5 to 1.75 mm from the burn edge. As at the 1-hour time point, progression of interstitial cell necrosis mirrored that seen in the epidermis, extending to approximately 1.2 mm from the burn edge by 4 hours post-burn.

A gradient of HMGB1 staining patterns was also observed at 4 hours post-burn. Extracellular and perivascular HMGB1 staining adjacent to the burn edge (Fig. 6D) was decreased compared to 1 hour (Fig. 6A). The greatest intensity of perivascular HMGB1 was observed 0.8 mm away from the burn (Fig. 6E). Vascular endothelial cells further from the

burn edge displayed a brighter cytoplasmic HMGB1 signal, but a lesser degree of perivascular HMGB1 staining (Fig. 6F). Additionally, a perivascular neutrophilic infiltrate was observed at 1.2 mm away from the burn.

By 24 hours post-burn, vascular endothelial cell and interstitial cell necrosis had progressed across the entire zone of ischemia, and further changes in HMGB1 staining patterns were evident. Compared to 1 and 4 hours, extracellular HMGB1 staining was markedly reduced (Fig. 6G-I). The area adjacent to the burn (Fig. 6G) was almost completely devoid of HMGB1 staining, similar to burned tissue beneath the hot comb at 1 and 4 hours. Comparison of all other zones showed a substantial decrease in extracellular HMGB1 staining at 24 hours compared to 4 hours post-burn (compare Fig. 6G to D, H to E, I to F). Additionally, a perivascular, mononuclear infiltrate was observed from 1.0 mm to 2.0 mm away from the burn (Fig. 6I).

When the results from HMGB1 changes in endothelial cells (Fig. 6) were statistically analyzed we found that the percent cell necrosis within 0.25×1 mm areas at progressive distances from the burn edge showed a statistically significant increase among the 1, 4, and 24-hour post-burn time points ($P < 0.001$) (Fig. 7). Thus, quantification confirmed the qualitative results shown in Figure 6.

Apoptosis

Burned Tissue Below the Hot Comb—No positive CC3a staining of keratinocytes, interstitial cells, or vascular endothelial cells was observed.

Zone of Ischemia Lateral to the Hot Comb—At 1 hour post-burn, no positive CC3a staining of keratinocytes, interstitial cells, or vascular endothelial cells was observed (Fig. 8B). At 4 hours post-burn, no positive CC3a staining was observed in the epidermis, while a few isolated interstitial cells showed positive CC3a staining indicative of cell apoptosis further than 1.25 mm from the burn. No interstitial cell apoptosis occurred closer to the burn, and no vascular endothelial cells apoptosis was observed at 4 hours (Fig 8C). By 24 hours post-burn, a band of cells with positive CC3a staining was observed deep to the area of necrosis, indicating a large number of apoptotic cells at the interface of necrotic and viable tissue (Fig. 8D). Both interstitial cells and vascular cells at this interface displayed positive CC3a staining.

Discussion

A satisfactory explanation of burn injury progression should provide a step-by-step account of the molecular and cellular events, set in motion by the initial thermal insult, that ultimately result in tissue death in the zone of ischemia. An important step in building such an explanation is to accurately characterize the fate of cellular constituents of periburn tissue that becomes grossly necrotic during the process of burn injury progression. It is important not to confuse the concepts of gross tissue necrosis diagnosed clinically with the mechanism of underlying cell death, which can occur by either cellular necrosis or apoptosis.

The results of our study indicate that necrosis is the most prominent mechanism of cell death in burn injury progression. HGMB1 extrusion from cell nuclei to the extracellular space provides an objective means of identifying cell necrosis, and staining patterns observed in our model demonstrate that a wave of necrosis progressed across the zone of ischemia from the zone of coagulation over time.

Cellular necrosis in the zone of ischemia was seen as early as 1 hour post-burn, and a substantial extension of necrosis into the zone of ischemia occurred between 1 and 4 hours.

While conversion of the zone of ischemia to frank tissue necrosis has been described as occurring over the first 48-72 hours following a burn, our data indicate that irreversible changes occur much earlier at the cellular level that only later become manifest as grossly visible tissue necrosis. A 0.75 mm progression of vascular endothelial cell necrosis occurred between 1 and 4 hours post-burn. In a superficial partial-thickness burn such extension vertically would likely mean the difference between spontaneous healing and conversion to a full thickness injury. Furthermore, our results imply that vascular endothelial cells may be more susceptible to death by burn injury progression and are likely key components of conversion of the zone of ischemia to frank tissue necrosis. The rapid extension of injury at the cellular level indicates that early intervention is likely necessary to limit burn injury progression.

While the presence or absence of nuclear HMGB1 staining can be used to clearly distinguish necrotic from non-necrotic cells, we believe that attention to specific patterns of extracellular HMGB1 staining provides additional information about the timing of necrosis. A pattern of intense cytoplasmic HMGB1 staining with leakage across indistinct cell borders was consistently observed at the conversion zone between necrotic and viable cells. This pattern of staining appears to indicate the “leading edge of tissue necrosis,” consisting of cells that are either caught in the act of undergoing necrosis or became necrotic a short time before biopsy. Vascular endothelial cells on this leading edge displayed the greatest amount of perivascular HMGB1 staining, evidence of HMGB1 recently spilled from necrotic cells. In addition, we consistently observed a time-dependent decrease in HMGB1 staining of necrotic tissue.

Based on the above observations we propose a definition of distinct temporal, pathological signs of cell necrosis based on specific patterns of cellular HMGB1 staining:

1. *Early*: A staining pattern characterized by absent nuclear HMGB1 staining, intense cytoplasmic HMGB1 staining, and a large amount of extracellular HMGB1 staining. Cells with this staining pattern are in the act of undergoing necrotic death or have done so very recently.
2. *Intermediate*: A staining pattern characterized by absent nuclear HMGB1 staining, poorly defined cell borders without cytoplasmic HMGB1 staining, and a lesser amount of interstitial HMGB1 compared to *early* necrosis. Cells with this staining pattern have likely undergone necrosis within the past several hours.
3. *Advanced*: A staining pattern characterized by a near absence of HMGB1 staining in the tissue, indicating that cellular necrosis occurred many hours to days prior to biopsy.

While prior investigators have proposed that apoptosis plays a significant role in burn injury progression³⁻⁵, our results indicate that apoptosis does not play a role in the death of keratinocytes, interstitial cells, and vascular endothelial cells in the zone of ischemia at early (1-4 hour) post-burn time points. Rather, apoptosis is a later phenomenon at 24 hours and is responsible for cell death at the furthest reach of burn injury progression. We attribute differences in our findings regarding apoptosis in burn injury progression to our use of a controlled experimental model with careful morphometric analysis.

A priori one might expect apoptosis and necrosis to evolve similarly in the horizontal and vertical direction; however, apoptosis and necrosis are spatiotemporally divergent around myocardial infarcts and cerebral vascular injuries, with necrosis appearing centrally within a few hours and apoptosis appearing peripherally over hours to days.^{21, 22} Thus, our finding of dissimilar spatiotemporal appearance of necrosis and apoptosis during burn injury progression parallels findings in myocardial infarcts and cerebral vascular injuries.

Furthermore, the appearance of this apoptotic band deep in the zone of ischemia at 24 hours post-burn may prove to be clinically significant as vertical progression can lead to the conversion of mid-dermal burns to full-thickness burns.

We believe that the present study is an important and necessary step towards an understanding of the cellular and molecular events underlying burn injury progression. Nonetheless, a number of factors remain to be addressed in future experiments. Firstly, our hot comb burn model creates zones of ischemia lateral to burns, which allows us to assess horizontal progression of burn injury. While horizontal extension of human burn injuries does occur, the most important consequences of burn injury progression result from vertical extension of burn depth. While the present hot comb model serves as a useful model of the zone of ischemia, it will be important to replicate these experiments in a vertical model of burn injury progression. Secondly, additional biomarkers are needed to identify the types of interstitial cells present at different time points after burn injury. Interstitial cells would include fibroblasts, dermal dendritic cells, myocytes and blood leukocytes. While these endeavors are beyond the scope of this manuscript, we believe that such studies are critically important to further define burn injury progression. In future research efforts we aim to interrogate single tissue specimens with as many as 34 biomarkers to define not only the interstitial cell types but also their activation state. This will be done in collaboration with GE Global Research who has created a system of in situ multiplexing that can accomplish this goal.

In conclusion, the findings of our study indicate that necrosis is the most prominent mechanism of cell death in burn injury progression, with evidence of significant extension of necrotic cell death in the zone of ischemia between 1 and 4 hours post-burn. Apoptosis did not play a role in early burn injury progression, while cells at the interface of necrotic and viable tissue underwent apoptosis at 24 hours post-burn. An implication of these findings is that intervention within the first 4 hours following injury is likely necessary to limit irreversible cellular changes that underlie burn injury progression.

In addition, HMGB1 provides an objective and reliable means of detecting cellular necrosis, especially in the absence of obvious cellular architectural changes. Attention to subtleties of HMGB1 staining patterns evidences distinct temporal, pathological signs of necrosis. We believe that these distinctions may prove to be significant in the search for mechanisms of burn injury progression. Elucidation of the causal role of a particular pathological process, such as oxidative stress, in burn injury progression requires the in vivo temporal relation of that process to cell death. Using the above distinctions in HMGB1 staining patterns, we can infer that processes temporally correlated with an *early* HMGB1 pattern are likely causal factors in burn injury progression, while processes associated with an *advanced* or *late* HMGB1 pattern are likely a result of cellular necrosis during burn injury progression. It is our hope that these findings will facilitate delineation of a step-by-step explanation of the pathophysiology of burn injury progression.

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Figure 1.

A) Burns are created with a 150 g brass comb with 4 10×25 mm prongs separated by 5 mm interspaces. **B)** Appearance of burn wound immediate after application of the hot comb. 8 mm punch biopsies encompass the 5 mm interspace (zone of ischemia) with 1.5 mm of burned tissue on either side. **C)** Slide-mounted tissue sample illustrating orientation on slide relative to biopsy placement. For each specimen, a single burn was chosen for analysis with its corresponding zone of ischemia. Boxes display 0.25 mm wide $\times$ 1.0 mm deep zones used to quantify endothelial cell necrosis.

Figure 2. HMGB1 controls. **A)** Unburned dermis stained for HMGB1. The arrow indicates a vascular endothelial cell displaying nuclear HMGB1 staining indicative of cell viability. **B)** Unburned epidermis stained for HMGB1. Keratinocytes display nuclear HMGB1 staining indicative of cell viability.

Figure 3.

Vascular endothelial cell staining of sequential sections. **A-C)** Tissue from the zone of ischemia stained for Laminin, CD31, and HMGB1, respectively. Notice that necrotic vascular endothelial cell nuclei displayed in Panel C have lost nuclear HMGB1 staining, in comparison to viable endothelial cells shown in Fig. 2A.

Figure 4. Patterns of epidermal HMGB1 staining. **A)** Absence, **B)** Diffuse extracellular staining, **C)** Concentrated extracellular and cytoplasmic staining, **D)** Nuclear staining without extracellular staining.

Figure 5.

HMGB1 staining of burn and interspace at 1, 4, and 24 hours post-burn. Individual panels depict 0.5 mm increments moving from the burn into the zone of ischemia to the right. **A)** Directly below the hot comb at 1 hour, **B-E)** 0.5 mm intervals moving away from the burn at 1 hour, **F)** Directly below the hot comb at 4 hours, **G-J)** 0.5 mm intervals moving away from the burn at 4 hours, **K)** Directly below the hot comb at 24 hours, **L-O)** 0.5 mm intervals moving away from the burn at 24 hours. Arrows mark the progression of epidermal necrosis over time as indicated by loss of keratinocyte nuclear HMGB1 staining. Boxes mark high-powered fields displayed in Figure 6.

Figure 6.

Vascular endothelial cell HMGB1 staining at 1, 4, and 24 hours post-burn. **A-C)** 1 hour post-burn: high-powered views of Fig. 5B-D, respectively. **D-F)** 4 hours post-burn: High-powered views of Fig. 5G-I, respectively. **G-I)** 24 hours post-burn: High-powered views of Fig. 5L-N, respectively. Arrows mark vascular endothelial cells with loss of nuclear HMGB1 staining and leakage of HMGB1 into the extracellular space indicative of necrosis.

Figure 7. Spatiotemporal variation in endothelial cell necrosis. A statistically significant increase in percent endothelial necrosis was observed among the 1, 4, and 24-hour post-burn time points (mean $\pm$ SE, $n = 3$ for each time point, $P < 0.001$).

Figure 8. CC3a staining at 1, 4, and 24 hours post-burn. **A)** Unburned control, **B)** 1 hour, **C)** 4 hours, **D)** 24 hours. A band of apoptotic cells was observed deep to the area of necrosis, at the interface of necrotic and viable tissue. Panels display the degree of apoptosis for a burn and adjacent 2.5 mm zone of ischemia that spans one half of the entire interspace.