Thermal injury effects on intestinal crypt cell proliferation and death are cell position dependent

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Varedi, Maryam, Rebecca Chinery, George H. Greeley, Jr., David N. Herndon, and Ella W. Englander. Thermal injury effects on intestinal crypt cell proliferation and death are cell position dependent. Am J Physiol Gastrointest Liver Physiol 280: G157–G163, 2001.—We examined the effects of thermal injury on intestinal epithelial cell proliferation and death. We recorded histologically identifiable mitotic and apoptotic crypt cells in relation to cell position after a 60% full thickness cutaneous thermal injury in the rat. The injury significantly reduced mitosis (0.53 ± 0.11 vs. 1.50 ± 0.70, P < 0.05) at cell positions 4–6, stem cells, 6 h after injury. A similar reduction in mitosis (1.13 ± 0.59 vs. 3.50 ± 0.80, P < 0.05) was observed at higher cell positions 7–9 12 h after injury, indicating a positional cell shift. In addition, a significant increase in the number of apoptotic bodies occurred at cell positions 7–9 (2.32 ± 0.87 vs. 0.13 ± 0.22, P < 0.05) and 10–12 (2.2 ± 0.12 vs. 0.00, P < 0.05) 6 h after injury. Thermal injury-induced alterations in mitotic and apoptotic activities were transient since crypts recovered with a moderate increase in mitotic activity 24 h after injury. In control and thermal-injury rats 24 h after injury, crypt cell mitosis and apoptosis did not differ significantly. This demonstrates that cutaneous thermal injury causes a transient suppression of mitosis as well as induction of apoptosis in a cell position-dependent manner in the small intestinal crypt.

burn; trauma; small intestine; mitosis; apoptosis; proliferation potential

THERMAL INJURY IS ASSOCIATED with a dramatically altered function in multiple organs (1, 45). Despite intensive research into the pathophysiology of thermal injury (32, 42), the cellular and molecular mechanisms involved in the adverse effects of thermal injury are poorly understood. Evidence indicates that thermal injury leads to a release into the systemic circulation of a factor(s), known as “burn toxin,” that can compromise tissue function (2, 28). Earlier, we reported in vitro findings showing that a thermal injury-induced circulating factor(s) causes significant alterations in morphology and a diminished growth rate and migration (46) of rapidly proliferating rat intestinal epithelial cells (IEC-6) (40). Other investigators have reported that sera collected from thermal-injury patients induce similar alterations in morphology and growth of dermal fibroblasts and keratinocytes (21). It has also been shown, however, that other slower proliferating cell types, such as liver and cardiac cells, in vivo, are not equally affected by thermal injury (23). Hence, the adverse effects of thermal injury appear to be related to the proliferative growth characteristics of the affected tissue.

Intestinal mucosal cells have one of the highest turnover rates in the body (39) and are exceptionally responsive to various types of stress, including thermal injury (3–5, 14, 24, 30, 35, 37). Thermal injury induces intestinal atrophy and causes marked alterations in the morphology, growth, and function of the small intestinal epithelium (5, 7, 9, 11). Post-thermal injury atrophy of the intestinal mucosa was first described based on a decrease in the mucosa weight (7) and a reduced intestinal cell proliferation, as demonstrated by diminished DNA and protein synthesis (7). These earlier studies, however, did not address the question of which mucosal cells are the primary targets for thermal injury.

All intestinal epithelial cells are derived from a small population of cells situated in the crypt base (8, 35, 37, 48). Cells proliferate in the crypts, differentiate as they migrate to the villi, and reach the villi tips, where they are shed into intestinal lumen. Thus changes in villus morphology or function can be a consequence of altered cell production in the crypt. In an earlier study, we used the bromodeoxyuridine pulse-chase method to show that thermal injury disrupts crypt cell proliferation (46).

A unique feature of the intestinal mucosa is that the proliferation potential and injury susceptibility of cells are related to cell position (38). Crypt cells, at specific positions, possess a distinct proliferation potential (35, 37) and appear to differentially detect the magnitude and the nature of insult (16, 17). In the present study, we used a thermal injury rat model to examine the hypothesis that thermal injury disrupts intestinal mu-
cosa homeostasis by targeting highly proliferative cells in the crypt. We monitored mitosis and apoptosis in the small intestinal crypts after a cutaneous thermal injury in the rat and identified specific intestinal crypt cells whose proliferation and survival are affected by the injury. Our results reveal that thermal injury reduces mitosis and induces apoptosis selectively in the proliferative compartment of the crypt at specific cell positions.

MATERIALS AND METHODS

Materials. All reagents used for preparation of paraffin blocks, histological sections, and hematoxylin and eosin staining were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma (St. Louis, MO). The apoptosis detection system kit for the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was purchased from Promega (Madison, WI).

Thermal injury model. Adult male Sprague-Dawley rats (300–400 g) were used in this study. The thermal injury model protocol was approved by the Institutional Animal Care and Use Committee of The University of Texas Medical Branch. A standardized cutaneous thermal injury was done according to a method described originally by Walker and Mason (47) and modified by Herndon et al. (15). In brief, rats were anesthetized by an intraperitoneal injection of pentobarbital (Nembutal; 30 mg/kg body wt) and given an intramuscular injection of a pain killer (butorphanol, Stadol; Bristol-Myers Squibb, Princeton, NJ; 0.6 mg/kg). The abdomen and back were then shaved, and the rats were secured into a plastic mold before exposure to scalding water. The dorsum of each rat was immersed in 96°C water for 10 s and then given an intraperitoneal injection of 10 ml of lactated Ringer solution; the rat’s ventrum was also exposed to 96°C water for 2 s. This procedure produces a full-thickness thermal injury of 60% total body surface area. Control (i.e., sham-injury) rats were prepared in an identical fashion, with the exception that they were immersed in ambient temperature water. After the thermal exposure, all rats were placed on a prewarmed electric blanket for 1–2 h and then transferred to individual metal cages and given food (Purina Lab Chow) and water ad libitum. Food intake of thermal injury rats was measured, and the control rats were pair fed according to the food intake of thermal-injury rats. The mortality rate was <5%.

Tissue collection. All rats were scalded between 8 and 10 AM and were killed by decapitation at the indicated times after injury. Rats in the 24-h group were killed in the morning, and those in the 6- and 12-h groups were killed at night. Specimens of the middle duodenum were rapidly harvested, and the intestinal contents were gently washed out of the intestinal lumen by two flushes of normal saline. For preparation of histological sections, the tissue was cut into small pieces and fixed for 24 h at 4°C in phosphate-buffered saline with freshly prepared 4% paraformaldehyde. Tissues were then processed, embedded in paraffin blocks, and sectioned at 4 μm using standard techniques. Sections were used for hematoxylin and eosin staining or in situ detection of apoptotic cells by TUNEL assay.

Scoring of mitosis and apoptosis. Intestinal tissue cross-sections were prepared such that crypts appear longitudinally and cell positions in the crypts can be outlined. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin using a standard protocol. Well longitudinally sectioned crypts were selected so that Paneth cells could be identified at the base of crypts and the crypt lumen was visible. Cell positions in the crypts were determined according to the published methods established by Potten et al. (36, 38). Briefly, from the base to the top, cells in each crypt column were divided into eight zones (3 cells/zone), and cells were numbered up to the 24th position (see Fig. 1). Position 1 was given to the cell situated at the very base, a Paneth cell, in the column, and position 24 was given to the cell situated at the crypt-villus junction, which represents the limit of occurrence of mitosis and apoptosis (38). The shape and integrity of crypt cells were examined at ×400 magnification. For each animal, at least 50 crypts were scored, and the number of mitotic cells and apoptotic bodies were obtained with respect to cell position. Distribution of mitotic or apoptotic bodies is quoted as percentage of total crypt cells for each zone. Out of the 6–10 rats in control or thermal-injury group that were killed at each time point, tissue sections of three randomly selected rats were used for scoring of mitosis and apoptosis.

TUNEL assay. Apoptotic cells/bodies in histological sections (4 μm) of the small intestine collected from control or thermal-injury rats were detected by a TUNEL technique described by Gavrieli et al. (12). The ends of fragmented DNA in apoptotic cells were tagged by terminal deoxynucleotidyl transferase-mediated incorporation of biotinylated dUTP nucleotide. Incorporated nucleotides were then visualized by horseradish peroxidase-labeled streptavidin in the presence of diaminobenzidine substrate and hydrogen peroxide. The protocol provided by the manufacturer (Promega) was followed. Cells stained positive for TUNEL were counted in a

![Fig. 1. Schematic representation of a longitudinal section of an intestinal crypt. Cells were numbered from the base, positions 1 through 24, according to the standard method described by Potten et al. (36, 38). PC, Paneth cell; position 1; ST, stem cell positions; PR, proliferative region; PMR, postmitotic region.](http://ajpgi.physiology.org/)
blinded fashion in randomly selected histological sections prepared from intestinal tissue of six rats in each group at each time point.

**Statistical analysis.** Regional differences in mitosis and apoptosis were analyzed by using ANOVA for a two-factor factorial experiment; the two factors were treatment (thermal injury or sham injury) and tissue harvest time (6, 12, or 24 h). For multiple comparisons, the least significant difference procedure was used with Bonferroni adjustment. The results represent means ± SD. P < 0.05 was considered statistically significant.

**RESULTS**

**Thermal injury reduces mitotic activity in rat intestinal crypts.** Evaluation of mitotic activity throughout the crypt proliferative region of the small intestine in both control and thermal-injury rats revealed discrete spatial differences (Table 1). Cells in the midcrypt zones are highly proliferative, and cells in the top crypt zones are postmitotic. We found that thermal injury inhibits mitosis in proliferating cells situated at specific positions in the crypt. Most zones in the proliferation region of the crypt in thermal-injury rats showed a reduction in mitotic figures 6 h after injury compared with those of control rats (Fig. 2). However, mitotic activity in different zones is not equally affected by thermal injury. Marked reductions at 6 h are observed in zones 2 and 4, but the reduction is statistically significant exclusively in zone 2, cell positions 4–6 (0.53 ± 0.11 vs. 1.50 ± 0.70). Reduced mitotic activity persists for at least 12 h after injury, with a significant reduction in zone 3, cell positions 7–9 (1.13 ± 0.59 vs. 3.50 ± 0.80), indicating a positional shift. By 24 h after injury, a restoration in mitotic activity is seen in the affected zones since the mitotic numbers normalize at the respective cell positions. In addition, both control and thermal-injury rats show an increase in crypt

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**Table 1. Thermal injury reduces mitosis in the proliferation region of small intestinal crypts**

<table>
<thead>
<tr>
<th>Zone</th>
<th>Cell Positions</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1–3</td>
<td>0.33 ± 0.06 0.00 ± 0.00</td>
<td>0.07 ± 0.11 0.00 ± 0.00</td>
<td>0.07 ± 0.11 0.03 ± 0.06</td>
</tr>
<tr>
<td>2</td>
<td>4–6</td>
<td>1.50 ± 0.70 0.53 ± 0.11*</td>
<td>1.00 ± 0.20 0.47 ± 0.15</td>
<td>1.27 ± 0.38 0.97 ± 0.20</td>
</tr>
<tr>
<td>3</td>
<td>7–9</td>
<td>2.90 ± 1.05 2.60 ± 0.66</td>
<td>3.50 ± 0.80 1.13 ± 0.59*</td>
<td>3.07 ± 0.64 2.97 ± 0.76</td>
</tr>
<tr>
<td>4</td>
<td>10–12</td>
<td>2.00 ± 0.85 0.80 ± 0.20</td>
<td>1.03 ± 0.45 1.00 ± 0.70</td>
<td>1.60 ± 0.26 1.93 ± 0.42</td>
</tr>
<tr>
<td>5</td>
<td>13–15</td>
<td>0.67 ± 0.29 0.47 ± 0.11</td>
<td>0.43 ± 0.15 0.47 ± 0.15</td>
<td>0.97 ± 0.21†‡ 1.13 ± 0.42†‡</td>
</tr>
<tr>
<td>6</td>
<td>16–18</td>
<td>0.20 ± 0.17 0.33 ± 0.06</td>
<td>0.13 ± 0.11 0.13 ± 0.11</td>
<td>0.29 ± 0.10 0.10 ± 0.10</td>
</tr>
<tr>
<td>7</td>
<td>19–21</td>
<td>0 0 0 0</td>
<td>0 0 0 0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>8</td>
<td>22–24</td>
<td>0 0 0 0</td>
<td>0 0 0 0</td>
<td>0 0 0 0</td>
</tr>
</tbody>
</table>

Values are means ± SD given as % mitotic activity in different crypt zones in control (C) and thermal-injury rats (TI) at the indicated times after injury. At least 50 crypts were scored to determine the distribution of mitotic activity for each animal at each time point with respect to cell positions, yielding the total mitotic cells for the respective zone. Intestinal crypts of 3 rats were scored at each time point. *P < 0.05 vs. control; †P < 0.05 vs. rats in respective group 6 h after injury; ‡P < 0.05 vs. rats in respective group 12 h after injury.
mitotic activity in zone 5, cell positions 13–15, 24 h after injury. The increases in mitotic figures are statistically significant when compared with those of respective crypt zones in the rats examined at earlier time points.

**Table 2.** Thermal injury induces apoptosis in the proliferative region of small intestinal crypts

<table>
<thead>
<tr>
<th>Zone</th>
<th>Cell Positions</th>
<th>C</th>
<th>TI</th>
<th>C</th>
<th>TI</th>
<th>C</th>
<th>TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1–3</td>
<td>0.51±0.22</td>
<td>0.13±0.12</td>
<td>0.06±0.05†</td>
<td>0.09±0.08</td>
<td>0.03±0.05†</td>
<td>0.03±0.05</td>
</tr>
<tr>
<td>2</td>
<td>4–6</td>
<td>0.75±0.65</td>
<td>1.07±1.01</td>
<td>0.14±0.05</td>
<td>1.02±0.35</td>
<td>0.14±0.05</td>
<td>0.23±0.05</td>
</tr>
<tr>
<td>3</td>
<td>7–9</td>
<td>0.13±0.22</td>
<td>2.32±0.87†</td>
<td>0.20±0.05</td>
<td>0.93±0.44†</td>
<td>0.09±0.08</td>
<td>0.28±0.05†</td>
</tr>
<tr>
<td>4</td>
<td>10–12</td>
<td>0</td>
<td>0.49±0.25†</td>
<td>0</td>
<td>0.03±0.05</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>13–15</td>
<td>0</td>
<td>0.09±0.08</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>16–18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>7</td>
<td>19–21</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>8</td>
<td>22–24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are means ± SD given as % apoptotic activities in different zones of small intestinal crypts in C and TI rats at the indicated times after injury. At least 50 crypts were scored for each rat at each time point with respect to cell positions, yielding the apoptotic cells/bodies in total for the respective zone. Intestinal crypts of 3 rats were scored. *P < 0.05 vs. rats in control group; †P < 0.05 vs. rats in respective group 6 h after injury.

Thermal injury induces apoptosis in rat intestinal crypt: morphological analysis. Counts of apoptotic cells in the small intestinal crypt of control and thermal-injury rats at the indicated times after injury are shown in Table 2. A low level of apoptotic activity was detected in the base to midcrypt region in control rats. This basal level of apoptotic activity is known as “spontaneous apoptosis” and has been reported in humans and rodents under normal conditions (34). As shown in Table 2, thermal injury induces apoptosis in zones 2–5, cell positions 4–15, 6 h after injury. Statistical analysis revealed that the thermal injury-induced apoptosis is significant exclusively in zone 3, cell positions 7–9 (2.32 ± 0.87 vs. 0.13 ± 0.22) and zone 4, cell positions 10–12 (0.49 ± 0.25 vs. 0.00). Up to a 17-fold increase in the number of apoptotic cells/bodies is observed at cell positions 7–9 in the crypt of thermal-injury rats 6 h after injury. Thermal injury-induced apoptosis significantly declined in zone 3, cell positions 7–9 (2.32 ± 0.87 vs. 0.93 ± 0.44) and zone 4, cell positions 10–12 (0.49 ± 0.25 vs. 0.14 ± 0.05) 12 h after injury. Although the elevated levels of crypt apoptotic activity in thermal-injury rats persist at 24 h, the number of apoptotic cells/bodies did not differ significantly in control and thermal-injury rats at 12 and 24 h after injury.

Large variations, in sham-treated rats, for apoptosis values occur at the 6-h postinjury time point in zones 1 and 2. We suspect that these effects are associated with acute stress generated in the course of the sham treatment, especially in view of the limited number of zones affected and their short duration.

**DISCUSSION**

Cutaneous thermal injury has been shown to disrupt homeostasis of multiple tissues (1, 32, 42, 45). In the small intestine, cutaneous thermal injury results in morphological, functional, and growth alterations of the mucosal epithelium (9, 29, 46). In fact, the intestinal mucosa provides a unique system for studying the cellular and molecular mechanisms involved in the effects of injury-induced stress on cell growth and death in vivo (20). The crypt region represents a series of cell lineages with different proliferation potentials and sensitivities to stress or injury with respect to cell position (16, 17, 39). In the present study, we measured mitosis and apoptosis in the crypt, on a cell-positional basis, and found that cells with a high proliferation potential are primary targets for thermal injury.

In this report, we show that thermal injury reduced mitosis in the rat small intestine. Morphological analysis of crypt cells indicated that crypt mitotic activity decreased throughout the proliferation zone. The maximal reduction was observed in cells located in zone 2, positions 4–6, at an early time (6 h) after injury and at a higher cell position, zone 3, cell positions 7–9, at a later time (12 h). This temporally related zone shift in depressed crypt cell mitosis has not been described previously for thermal injury. It may be due to the changing character of noxious, endogenous agents released locally and into the general circulation following thermal injury that influence intestinal cell proliferation. Our findings with mitosis resemble findings from earlier studies (16, 17, 39) demonstrating that the sensitivity of crypt cells to induction of apoptosis varies with respect to cell position.

Crypt stem cells possess an exceptionally high proliferation potential and play a central role in intestinal epithelial homeostasis (33, 34). Stem cells are also responsible for production of four intestinal epithelial cell types: enterocytes, enteroendocrine, Paneth, and goblet cells (8). In a previous study (46), we showed a significant reduction in villus goblet cell number and exhibited cytoplasmic shrinkage and nuclear condensation, canonical characteristics of apoptotic cells.
marked alterations in villus enterocyte shape 12 h after thermal injury. Our present findings show that the reduction in crypt mitotic activity precedes the alterations observed in the villus. Crypt cells proliferate in the crypts and, at a constant rate, migrate to the villi. Thus morphological alterations in villus cells most likely reflect a perturbation in cell proliferation in the crypt. The thermal injury-induced reduction in mitosis was transient. Other investigators have shown the largest elevation in intestinal permeability (5–7) and a disruption in the brush border cytoskeleton (11) 18 h after injury. Such mucosal alterations may alter gut nutrient absorptive capacity and have a primary role in the increase in gut permeability following a thermal injury (10, 19, 43). This increase in gut permeability may contribute to the translocation of bacteria and associated endotoxins and ultimately increase the risk of sepsis (31). Such adverse effects on intestinal function may reflect the altered mitotic and apoptotic activities in the crypt occurring at earlier times after thermal injury. Within 24 h, crypts recovered with a greater mitotic activity in the higher proliferation zone. Increased mitotic activity has been reported previously in crypts recovering from radiation or starvation-induced stress (3, 39). Food intake and luminal nutrients can drive mitotic activity in the crypt (3, 18). However, in this study, the increased mitotic activity observed is not related to altered food intake and luminal nutrients, since nutrient intake in thermal-injury and control rats was equivalent.

The mechanisms involved in regulation of epithelial homeostasis in the intestinal mucosa are not fully understood. Cell death by apoptosis may play a crucial role in crypt cell homeostasis (13, 25, 41). In rodents and humans, a small fraction of crypt cells in the small intestine undergo apoptosis. This basal level of apoptotic activity is known as spontaneous apoptosis, which occurs predominantly in stem cells, positions 4–6 (34, 35, 37, 48). In this study, thermal injury-induced apoptosis is observed in a slightly broader region; apoptotic bodies were observed at stem cell positions as well as higher cell positions. These results agree with the observations that apoptosis induced by cytotoxic insults occurs in the stem cell as well as in higher cell positions.
positions (16, 17). It is thought that all crypt cells are programmed to undergo apoptosis, but the program is inactive in the absence of an appropriate stimulus (26, 27). Our findings show that thermal injury triggers apoptosis primarily in cells situated at specific positions. Cell position seems to play a role in sensing the intensity and the nature of the injury (16, 17, 33). For instance, low- and high-dose radiation (27, 34) or cytotoxic and genotoxic chemicals (16, 17) induce apoptosis in crypt cells situated at different cell positions. This cell position-related response to cytotoxic insults has been linked to the cell growth characteristics and the stage of cell cycle (16, 17). In our laboratory, earlier in vitro experiments suggest that thermal injury can arrest intestinal epithelial cells at G$_{S}$/M and trigger apoptosis (unpublished data). A similar in vivo observation was reported by others (26, 27) with a different type of insult; radiation injury-induced apoptosis in intestinal crypts of p53-null mice was shown to be related to G$_{S}$/M phase arrest in cycling crypt cells. Whether crypt cells, whose apoptosis was activated by thermal injury, were at the same phase of the cell cycle and whether thermal injury-induced apoptosis is also p53-independent demands further investigation. Control rats also showed an increase in apoptotic activity in the crypt base 6 h after injury. Sham treatment-related stress may be responsible for this effect, since stem cells are extremely sensitive to various types of insults and are activated to undergo apoptosis even with minor and repairable damage (34).

In this study, we used TUNEL as a complementary assay to evaluate apoptotic activity in intestinal crypts after thermal injury. The findings for the TUNEL assay at 6 h after injury are in agreement with the corresponding morphological analysis.

Thermal injury-induced alterations in mitotic and apoptotic activities were transient since crypts recovered with a moderate increase in mitotic activity 24 h after injury. This increase may not be stress related; it is plausible that increased mitotic activity is influenced by circadian rhythms. Circadian/diurnal rhythms may be associated with the increased mitotic activity observed in the respective crypt zone. Most proliferative cells in the body, gut epithelial cells in particular, exhibit circadian/diurnal rhythms in proliferation (22, 44). For the gastrointestinal tract, the maximum proliferative activity is recorded in the early morning hours, 3–6 AM (38). Whether chronological response is specific to cells at the defined cell positions observed in this study remains to be determined.

In summary, the present study shows that thermal injury disrupts epithelial homeostasis in the gut mucosa by an inhibition of mitosis and induction of apoptosis in the crypt at specific cell positions and that thermal injury-induced functional and morphological alterations in the villi reported in previous studies are, at least in part, related to imbalanced cell production and cell death in the crypt. The data presented here clearly show that cells with a high proliferation potential are the most responsive cells to thermal injury. Our data also provide new insights into the selective cytotoxic effects of cutaneous thermal injury on certain cell types within the body.

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