A thermal injury-induced circulating factor(s) compromises intestinal cell morphology, proliferation, and migration

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Varedi, Maryam, George H. Greeley, J r., David N. Herndon, and Ella W. Englander. A thermal injury-induced circulating factor(s) compromises intestinal cell morphology, proliferation, and migration. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G175–G182, 1999.—The effects of a 60% body surface area thermal injury in rats on the morphology and proliferation of the epithelium of the small intestine and the in vitro effects of serum collected from scalded rats on intestinal epithelial cells were investigated. Scald injury caused significant reductions in duodenal villus width and crypt dimensions, villus enterocytes changed in shape from columnar to cuboidal, and the number of goblet cells decreased. The proportion of bromodeoxyuridine-labeled S phase cells in crypts was also diminished. In vitro, incubation of intestinal epithelial cells (IEC-6) with scalded rat serum (SRS) collected at either 12 or 24 h after injury caused a disruption in the integrity of the confluent culture and induced the appearance of large denuded areas. SRS also decreased DNA synthesis and delayed wound closure in an in vitro wound-healing model. The thermal injury-induced changes in intestinal mucosal morphology and epithelial cell growth characteristics described in this study may underlie, in part, the mechanism(s) involved in the diminished absorption of nutrients, increased intestinal permeability, and sepsis in patients with thermal injury.

Materials and Methods

Materials. The IEC-6 cell line was purchased from American Type Culture Collection (Manassas, VA) at passage 13. In this study, we used passages 18–26. The IEC-6 cell line was derived originally from normal rat intestinal crypt cells (37). Intestinal epithelial cells are not transformed and retain the undifferentiated characteristics of epithelial stem cells. The 5-bromo-2'-deoxyuridine (BrdU) incorporation assay kit was purchased from Amersham (Arlington Heights, IL). Media, fetal bovine serum (FBS), antibiotics, insulin, and sodium pyruvate were from GIBCO BRL (Grand Island, NY). The neutralizing antibodies to transforming growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α) were obtained from R&D Systems (Minneapolis, MN) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. IGF-I was a gift of Genentech (South San Francisco, CA).

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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 burn of 60% total body surface area. Control (i.e., sham thermal injury) rats were prepared in an identical fashion, with the exception that they were immersed in ambient temperature water. After the injury, 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 thermally injured rats was measured, and the sham scald injury rats were pair fed according to the food intake of thermally injured rats. The mortality rate was <5%.

Serum and tissue specimen collection. Three hours before death, BrdU (175 mg/kg body wt) was given intraperitoneally. Rats were killed by decapitation at the indicated times. Trunk blood samples were collected individually in glass tubes and allowed to clot at 4°C. Sera were then harvested, aliquoted, and stored at −80°C. Serum samples collected from rats in the same group were pooled. Sera collected from control rats and stored at −80°C were thawed and harvested after a brief exposure to trypsin (0.1% wt/vol) and EDTA (0.02% wt/vol). Cells were then plated at 5–5.8 × 10^5 in 0.5 ml of test medium. To examine the effects of exogenous IGF-I, IEC-6 cells were grown in DMEM supplemented with 5% FBS, 4 µg/ml bovine insulin, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 4 mM glutamine. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were subcultured every 3–5 days with one to two changes of medium. For the described experiments, confluent cells were washed with PBS and harvested after a brief exposure to trypsin (0.1% wt/vol) and EDTA (0.02% wt/vol). Cells were then collected in complete fresh medium and centrifuged at 800 rpm for 5 min at room temperature. Cell pellets were resuspended in a small volume (500 µl) of serum-free medium, and the cell numbers were determined. Cells were then plated at 5–5.8 × 10^5 cells per 100-mm tissue culture dish in 7 ml of test medium containing 4% CRS (i.e., sham-scald injury) or SRS and 1% FBS. Changes in cell growth characteristics were monitored by light microscopy for 48 h. For BrdU incorporation experiments, cells were plated in a four-well chamber slide system (Fisher Scientific) at 2 × 10^5 in 0.5 ml of test medium. To examine the effects of exogenous IGF-I, IEC-6 cells were seeded in six-well plates at 0.8 × 10^5 with 2.5 ml of test medium in the absence or presence of IGF-I (80 ng/ml) for 62 h. At the end of this incubation period, cells were counted. In the TNF-α and TGF-β1 neutralization experiments, IEC-6 cells were cultured in the same manner in the presence of TNF-α neutralizing (10 ng/ml) or TGF-β1 (100 ng/ml) neutralizing antiserum.

Dimensions of villi and histological evaluations. Tissue sections (4 µm) of small intestine were deparaffinized, rehydrated, and stained with hematoxylin-eosin or alcian blue using conventional protocols. The hematoxylin-eosin-stained sections were then photomicrographed, and the length and width of the villi were measured in the prints. Evaluations of morphological alterations in serial sections of duodenal tissue (at least 25 sections) from 6–10 animals were blinded. At least 15 villi in three different locations of each section (5 villi/location) from three rats in each group were examined for the quantitative analyses of villus dimensions. For quantitative analysis of the goblet cells, alcian blue-stained cells were counted in 10 villi at two different locations of each section. Tissue sections from at least three animals were examined in each group. For the quantitative analysis of BrdU-positive cells in crypts, tissue sections of three rats in each group were examined and positive cells of at least 20 crypts (2 locations/section, 10 crypts/location) were counted.

BrdU incorporation. IEC-6 cells were grown on the chamber slide system (Fisher Scientific) in the presence of 4% CRS or SRS for 18, 24, or 42 h. Cells were then exposed to BrdU (10 µM) for 2 h at 37°C. Cultures were then extensively washed with PBS, fixed for 18 h at 4°C in 4% fresh PBS-buffered paraformaldehyde (pH 7.4), and processed for BrdU staining. Briefly, after two 15-min washes in PBS, slides were treated with a PBS-based solution containing 0.1% trypsin and 0.1% CaCl₂ for 10 min at 37°C followed by incubation in 4 N HCl for 20 min at room temperature to produce DNA that is accessible to the antibody. Cells were then washed for 20 min with PBS, and nonspecific antibody binding was blocked by incubating for 1 h with 4% BSA with 0.05% Tween 20 at room temperature. A mouse monoclonal anti-BrdU antibody and alkaline phosphatase detection system were used to detect the incorporated BrdU. Positive cells were counted (blinded) in five microscopic fields (>40) and scored as percentage of the total cell count. For in vivo evaluation of BrdU incorporation, 4-µm sections of small intestine were deparaffinized in xylene, rehydrated in graded ethanols, washed in PBS, and processed for BrdU immunohistochemistry as described for the IEC-6 cells.

Cell migration assay. IEC-6 cells were grown on sterilized glass slides in regular medium. At confluence, cultures were wounded using a surgical blade (28). Three wounds, ~4 mm wide, were uniformly generated in each culture. Immediately after they were wounded, cells were washed with serum-free medium and the medium was replaced by fresh medium containing 4% CRS or SRS and 1% FBS; the equality of the wound dimensions was verified microscopically. At the indicated times, the cultures were examined using a phase-contrast microscope, and the wound closures were recorded by photomicrographs. The magnitudes of wound closure were determined by measuring the distance between the wound edges on the photographs and calculated as a percentage of the original wound size. Three wounds were measured for each treatment, and an average of three measurements per wound was used to calculate percentage of wound closure.

Statistical analysis. Differences in the villus dimensions were analyzed by one-way classification ANOVA with sub-samples. The in vivo and in vitro BrdU incorporation data were analyzed using one-way ANOVA and ANOVA for a two-factor factorial experiment, respectively. The goblet cell data were analyzed by ANOVA for a two-factor factorial experiment; the two factors are treatment (treated and control) and time after the treatment (12 and 24 h). The cell migration assay results were analyzed using ANOVA for a two-factor experiment with repeated measures for time. The results represent means ± SE. P < 0.05 was considered statistically significant.

RESULTS

Thermal injury-induced changes in intestinal mucosal morphology. To examine the effects of a thermal injury on small intestine mucosal morphology, hema-
toxylin-eosin-stained duodenal coronal sections were examined by light microscopy (Fig. 1A). Sections of control (sham thermal injury) rats at 12 h showed relatively wide villi with narrow spaces between adjacent villi, elongated columnar cells, and numerous goblet cells (Fig. 1A, left). In contrast, the duodenal mucosa from thermally injured rats at 12 h showed conspicuous morphological changes. Duodenal villi of thermally injured rats showed primarily narrow villi [1.5 ± 0.09 (control) vs. 1.0 ± 0.07 (thermal injury) arbitrary units; P < 0.05] and wide intervillous spaces (Fig. 1A, right). Epithelial columnar enterocytes of thermally injured rats became smaller and cuboidal in shape. A significant decrease in the number of goblet cells was evident 12 h [257 ± 16 (control) vs. 188 ± 8 (burn) goblet cells/10 villi] and 24 h [303 ± 26 (control) vs. 198 ± 10 (burn) goblet cells/10 villi] after thermal injury. Cross-sectional dimensions of crypts were substantially reduced, showing primarily a decreased crypt width in thermally injured rats (Fig. 1A, right).

Intestinal crypt cell proliferation was evaluated by examining the number of cells that incorporate BrdU (Fig. 1B). In thermally injured rats (right), BrdU incorporation into crypt cells was significantly reduced 12 h after injury, with a limited number of cells per crypt incorporating BrdU in contrast to a near complete staining of crypt cells in control rats (left). The number of BrdU-positive crypt cells in thermally injured rats

![Fig. 1. In vivo effects of a thermal injury on small intestinal mucosal morphology. A: photomicrographs show hematoxylin-eosin-stained duodenal coronal sections of control (left) and thermally injured rats (right). Sections of control rats (sham thermal injury) at 12 h showed wide villi with narrow spaces between adjacent villi, elongated columnar cells, and robust crypts (left). Duodenal mucosa of thermally injured rats (right) showed conspicuous morphological changes at 12 h. Villi were primarily narrow with wide intervillous spaces, and epithelial columnar enterocytes became smaller and cuboidal in shape. Cross-sectional dimensions of crypts were substantially reduced, showing primarily decreased crypt width. B: reduced crypt cell proliferation in thermally injured rats. Photomicrographs of 5-bromo-2’-deoxyuridine (BrdU)-labeled duodenal crypts are shown for control (left) and thermally injured rats (right). Crypts of control rats showed a large proportion of cells incorporating BrdU (left), whereas, in thermally injured rats, the number of crypt cells in the S phase of the cell cycle was markedly reduced. Number of BrdU-positive cells in control and thermally injured rats was 174 ± 4 and 99 ± 8 positive cells/10 crypts (P < 0.05), respectively.](http://ajpgi.physiology.org/)}
was significantly lower than that in control rats [99 ± 13 (thermal injury) vs. 164 ± 11 (control) positive cells/10 crypts; n = 3].

Thermal injury serum induced changes in intestinal monolayer morphology. Dramatic changes in normal monolayer morphology (Fig. 2A) were observed in cells cultured for 30–48 h in the presence of 4% SRS (Fig. 2, B–H). Scattered epithelial cells became rounded and lost contact with neighboring cells (Fig. 2B, arrow). Within 1 h after the appearance of rounded cells, areas denuded of cells were generated in their vicinity (Fig. 2, C and D, arrows). New denuded areas progressively developed adjacent to the original ones (Fig. 2, E and F). Rounded cells shrank and exhibited condensed

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Fig. 2. Effects of scalded rat serum (SRS) on IEC-6 cell monolayer culture morphology and integrity. Photomicrographs of normal IEC-6 cells (A) and IEC-6 cells showing the effects of SRS collected at 12-h postinjury (SRS-12) during 30–48 h of incubation (B–H). Arrows show the initiation of cells rounding at 30 h (B), progressive development of denuded areas (C–H), isolated rounded apoptotic-like cells (G), and clusters of morphologically altered, crowded cells with condensed nuclei detached from the dish after 40–48 h (H).
nuclei, suggestive of apoptosis (Fig. 2G, arrows). Although most rounded cells were gradually eliminated from culture, some remained isolated in the center of denuded areas (Fig. 2E, arrow). Additional rounded cells with condensed nuclei continued to appear (Fig. 2, F and G), and larger denuded areas continued to develop. Eventually, confluent cells became detached from the culture dish in the form of clusters of rounded cells (Fig. 2H, arrows). To determine whether the effect of SRS was dose dependent, incubation of IEC-6 cells with 1%, 2%, and 3% SRS was also monitored. The results (data not shown) revealed a dose-related effect in that 1% SRS did not cause any change in the monolayer, whereas 2% and 3% SRS caused slight and moderate disruptions, respectively, as larger denuded areas appeared. To discern burn-induced factors that underlie these alterations, we tested the effects of neutralizing TNF-α (10 ng/ml) or TGF-β1 (100 ng/ml) antibodies. Neutralization of endogenous TNF-α or TGF-β1 did not modify the adverse effects of SRS (data not shown).

Thermal injury serum inhibits IEC-6 cell proliferation. The effects of SRS on IEC-6 cell proliferation were assessed by the BrdU incorporation assay (Table 1). Cells were plated and grown for 18, 24, or 42 h in the presence of CRS or SRS and pulsed with BrdU for 2 h. The percentage of BrdU-positive cells plated with CRS did not vary with time. Cells plated with SRS for 18 h showed a significant reduction in the proportion of positive cells compared with the cells plated with CRS (16.9 ± 5.5% vs. 28.1 ± 3.3%). No significant differences were observed in the percentage of BrdU-positive cells in the cultures plated for 24 h or 42 h. SRS-12 and SRS-24 appeared to have similar effects on the proliferation of IEC-6 cells. Because circulating IGF-I levels are reduced after severe thermal injury, we examined whether addition of exogenous IGF-I (80 ng/ml) can influence IEC-6 cell growth in the presence of SRS (Table 2). The IEC-6 cell number increased to 381 ± 6% with 2.5 ml of test medium in the absence (−) or presence (+) of insulin-like growth factor (IGF-I) (80 ng/ml) for 62 h. At the end of this incubation period, cells were counted.

Thermal injury serum inhibits IEC-6 cell migration. The effects of scald injury rat serum on the migration of epithelial cells were examined in an in vitro model of wound healing (28), and cell migration was assessed by a quantitative analysis of the wound closure. Cells were incubated in culture medium, allowed to reach confluence, and then wounded by the “knife cut procedure.” Immediately after cells were wounded, media were replaced with media containing 4% CRS or SRS. Wound closure was measured at 1, 28, and 52 h after wounding (Fig. 3). At 1 h after wounding, no significant changes were observed in the migration of epithelial cells to the wounded area of the cultures containing CRS (A), SRS-12 (B), or SRS-24 (C). The wounds in the cultures containing CRS were closed by 67 ± 1.2% (D) at 28 h after wounding, and at 52 h (G) the wound closure was nearly complete (96 ± 4.0%) (Fig. 4). Wounds in cultures containing SRS-12 were closed by 54 ± 3.1% after 28 h (Fig. 3E) and by 86 ± 4.2% at 52 h after wounding (Fig. 3H). Wounds in cultures containing SRS-24 (Fig. 3F) were closed by only 40 ± 3.1% at 28 h after wounding and by only 78 ± 2.7% at 52 h after wounding (Fig. 3I). Statistical analysis showed that the wound closure process was significantly (P < 0.05) delayed at 28 h by SRS-12 and SRS-24. However, 52 h after cells were wounded, the wound closure was significantly delayed only by SRS-24. Although cells were seeded and allowed to reach confluence before exposure to SRS, small areas denuded of cells were observed in cultures containing SRS collected at 12 or 24 h. A majority of these loci recovered, with large cells gradually occupying denuded areas (Fig. 3, E and H). Similar large cells were often observed at the leading edge of wounds in cultures containing SRS (Fig. 3I).

**DISCUSSION**

The primary findings of this study are that substantial morphological and proliferative changes occur in the small intestinal mucosa after thermal injury and that serum harvested from thermally injured rats contains circulating factor(s) that can alter the growth characteristics of cultured intestinal epithelial cells. Specifically, the major changes in the duodenal mucosa after thermal injury are a decreased villus width, increased intervillus spacing, diminished crypt dimensions, and a reduced crypt cell proliferation. The column-
narrow epithelium also assumes a cuboidal appearance. In vitro, SRS disrupts the integrity of the confluent monolayer culture and inhibits proliferation and migration of cultured intestinal epithelial cells. The identity of the circulating factor(s) that compromises the intestine in vivo and intestinal epithelial cells in vitro, however, is not known.

Postburn intestinal mucosal atrophy has been proposed by other investigators, based on a decrease in the mucosa weight (7, 8, 32). The changes in the duodenal mucosa after thermal injury described in this study suggest that the previously reported postburn reduction in the mucosa weight may be due to both crypt and villus diminution. Our results showing a reduced BrdU incorporation into crypt cells concur and extend earlier reports of decreased intestinal mucosal weight, DNA, and protein content in thermally injured animals (7, 8, 19, 32). To our knowledge, this is the first report that showed by decreased BrdU incorporation a reduced intestinal crypt cell proliferation in thermally injured animals. We have also shown a significant reduction in the number of goblet cells, the major mucus-secreting cells in the intestine (39). Morphological changes in the small intestine epithelium may also occur due to the reduced enteral nutrition following thermal injury (3, 17, 20, 25). Enteral nutrition is a profound stimulus for maintenance of epithelial growth and mucosal integ-

Fig. 3. In vitro wound closure is delayed in the presence of SRS. Photomicrographs show the migration of IEC-6 cells to wounded regions of the monolayer culture. IEC-6 cells were plated in regular medium, grown to confluence, and then scraped by a surgical blade to generate a 4-mm-wide wound. Medium was replaced immediately after wounding with fresh medium containing control rat serum (CRS) (A, D, and G), SRS-12 (B, E, and H), or SRS collected at 24 h postinjury (SRS-24) (C, F, and I). Closure of the wounds was monitored and photographed at 1 h (A–C), 28 h (D–F), and 52 h (G–I) after wounding.

Fig. 4. Widths of healing wounds in the presence of SRS were measured (3 wounds for each treatment and 3 measurements per wound), and percent of wound closure is shown. Wounds in CRS-treated cultures were closed by 67 ± 1.2% at 28 h after wounding, and at 52 h the wound closure was nearly complete (96 ± 4.0%) (●). Wounds in cultures treated with SRS-12 were closed by 54 ± 3.1% after 28 h and by 86 ± 4.2% at 52 h after wounding ( ■ ). Wounds in cultures treated with SRS-24 were closed by only 40 ± 3.1% at 28 h after wounding and by only 78 ± 2.7% at 52 h after wounding (▲ ).

*Extent of wound closure for cells incubated with SRS-24 was statistically different at 28 and 52 h (P < 0.05) compared with cells cultured with CRS; wound closure for cells incubated with SRS-12 was statistically different at 28 h only.
riticy. However, the substantial changes in the duodenal villus/enterocyte morphology and intervillus spacing observed in this study cannot be attributed solely to reduced enteral feeding, since food intake was reduced marginally (data not shown). Moreover, in fasted or starved rats, the intestinal villi are merely shorter and the number of epithelial cells along the length of the villi is reduced.

In vitro, SRS disrupts the integrity of the confluent monolayer culture of intestinal epithelial cells, indicating that cell-cell and/or cell-matrix interactions are affected. The progressive nature for formation of the denuded areas by SRS in the confluent IEC-6 cell cultures implies that SRS activates release of a paracrine or autocrine effector from IEC-6 cells. SRS may have disrupted the IEC-6 monolayer indirectly by inducing changes in cell morphology (i.e., cytoskeletal organization), which then affects cell adhesion molecules. In an earlier study, Ezzell and co-workers (12) have shown a marked change in the organization of the brush-border cytoskeleton in the epithelial lining of the intestine following thermal injury. The finding that SRS does not impair the capacity of seeded IEC-6 cells to grow to confluence argues against the presence of a toxic substance in the serum or to the absence of an essential growth factor(s) in SRS. In the gut, a monolayer of intestinal epithelial cells confers a protective barrier that may be affected in a similar manner by burn-induced circulating mediators. The gut mucosal barrier often loses its integrity after burn injury and leads to sepsis. It is noteworthy that other protective barriers including the blood-brain barrier are compromised after severe burn injury and that the sera from burn patients show blood-brain barrier permeabilizing activity (31). The present in vivo and in vitro BrdU results indicate that endogenous factors that regulate intestinal epithelial homeostasis are also compromised. The finding of a depressed BrdU incorporation into IEC-6 cells by SRS stresses the systemic nature of this factor(s). Because mitotic activity in the crypts is decreased, cell migration from crypts to villi is presumably slower. A villus feedback system may sense that fewer cells are migrating up the villi; hence, the columnar epithelium assumes a cuboidal appearance to maintain a covered villi. A similar phenomenon has been observed for the radiation-damaged intestine (34).

To identify thermal injury-induced circulating factor(s) that may underlie the intestinal changes after burn, we tested the effects of neutralizing antiserum to TNF-α and TGF-β1. Systemic TNF-α levels surge after thermal injury (5, 33). A significant elevation in the circulating level of TGF-β1 after trauma is also reported (30). TGF-β1 is also a potential mediator, since TGF-β1 inhibits growth and promotes apoptosis in cells of epithelial origin (2, 21). Our findings suggest that neither of these factors is involved in the SRS-induced morphological alterations in IEC-6 cells. Other factors that inhibit intestinal epithelial cell proliferation that are also activated by thermal injury include the pro-inflammatory cytokine interleukin-1 (IL-1) (5, 11). Thermal injury-induced elevations of IL-1 can decrease epithelial proliferation by reducing serum levels of IGF-I (9, 13, 14, 24, 38, 46); the IGF-I system plays a major stimulatory role in gut epithelial growth and in gut barrier function (4, 9, 35, 42). In the presence of SRS but not CRS, the proliferative activity of IGF-1 on IEC-6 cells in vitro was blocked. The mechanisms involved in blockage of this effect by SRS remain to be determined. It is possible that IGF-1 binding proteins (IGFBPs) participate in this effect, since IGFBPs are known to sequester IGF-I (16). Although remote, another possible mechanism is that SRS stimulates the synthesis of IGFBP2 in IEC-6 cells. IEC-6 cells have been shown to produce IGFBP2 in culture and respond to it as a growth inhibitory factor (16). Alternatively, SRS may block the growth effects of IGF-I by downregulation of IGF-I receptors.

The hypothesis that a factor(s) released into the circulation during a thermal injury can affect intestinal epithelial cell proliferation and other growth-related processes has not been tested previously. To characterize such effects is important in light of the multiple adverse consequences of thermal injury on intestinal function in humans and of the intestinal changes described in the present study. It is noteworthy that in our study SRS was collected 12 or 24 h after thermal injury; other investigators have shown the largest elevations in intestinal permeability (6–8) and a disruption in the brush-border cytoskeleton (12–18 h after thermal injury). In summary, because multiple growth-related processes such as cell adhesion, proliferation, and migration are affected by SRS, it is likely that several factors or pathways are involved in the intestinal changes after thermal injury.

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