Role of TNF-α in gut mucosal changes after severe burn

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Received 10 April 2001; accepted in final form 13 March 2002

GUT MUCOSAL HOMEOSTASIS, and thus the morphological and functional integrity of the gut, is maintained by a balance between epithelial cell proliferation and cell death. Proliferation of gut epithelium occurs by mitosis in the intestinal crypts, whereas cell death occurs throughout the crypt and villus (19). Apoptosis is programmed death and removal of senescent or otherwise dysfunctional cells without inflammation. It can be considered an antagonistic and regulatory process to cellular proliferation by mitosis. Both apoptosis and mitosis are continually ongoing in live gut epithelium to maintain mucosal cellular balance. This delicate balance of mucosal cell mass can be influenced by exogenous and endogenous factors, such as nutritional depletion, chronic disease, or severe trauma (i.e., severe burn). We (19) previously showed increased gut epithelial cell death by apoptosis in the gut epithelium after severe burn, which was associated with mucosal atrophy. Potential mechanisms for these effects include the induction of epithelial cell death indirectly by relative hypoperfusion or directly through interaction of inflammatory mediators and their receptors located on gut epithelial cells. We (14) recently showed that burn-induced hypoperfusion of the gut is insufficient to induce apoptosis of gut epithelial cells. In other organs, programmed cell death can be induced by several membrane-bound ligand-receptor interactions, such as through Fas ligand (FasL)-Fas interaction or tumor necrosis factor-α-TNF receptor (TNF-α-TNFR) interaction (3). We therefore hypothesized that TNF-α-TNFR interaction activates apoptosis in small bowel mucosal cells after severe burn, and thus is a crucial element in changes in gut mucosa after severe burn.

MATERIALS AND METHODS

Adult male C57BL6 mice (Harlan Sprague-Dawley, Houston, TX) weighing 23 ± 2 g were housed individually in a temperature-controlled cubicle with a 12:12-h light-dark cycle. Mice were fed and received water ad libitum. The study was approved by the Animal Care and Use Committee of The University of Texas Medical Branch, Galveston, TX.

After 1 wk, the mice were randomly assigned to sham burn control, 30% total body surface area (TBSA) scald burn, and 30% TBSA scald burn with treatment by neutralizing hamster-anti-mouse-TNF-α antibody. Mice were anesthetized with methoxyflurane as inhalational agent (1–4%) and buprenorphine hydrochloride (0.1 mg/kg) given subcutaneously. The dorsum of the trunk was shaved, and a 30% TBSA burn was administered by placing the animals in a mold exposing an area of 4.2 × 2.9 cm of the back. The mold was placed in a column of 95 to 99°C steam for 6 s, which delivered a full thickness cutaneous burn demonstrated by histologic sections. Sham control animals were anesthetized, shaved, and placed in the mold without exposure to steam. Burned animals were resuscitated with 1 ml of 0.9% NaCl solution sq and 1 ml NaCl solution ip. Anti-TNF-α-treated animals received 200 μg neutralizing hamster anti-mouse-TNF-α antibody (BD Pharmingen, San Diego, CA) in 1 ml saline ip in addition to 1 ml resuscitation fluid sq. Mice were then returned to their cages. This time point was chosen in reference to our previously published findings (19) in which the maximum apoptotic reaction in small bowel was seen at 12 h. After sham burn or burn, all groups were given water ad libitum and fasted to avoid the confounding variable of dif-
ferent food intake in burned and unburned animals. Animals were killed at 12 h after injury by decapitation. The entire proximal segment was snap frozen in liquid nitrogen and stored at −80°C for future analysis. The cDNA reaction as well as the PCR were performed with an optimized buffer and enzyme system (Titan One Tube RT-PCR System; Roche, Indianapolis, IN) according to the manufacturer’s instruction. This system is designed to use avian myeloblastosis virus (AMV) RT for first-strand synthesis and the Expand high-fidelity blend of thermostable DNA polymerases, which consists of Taq DNA polymerase and a proof reading polymerase, for the PCR part. The reaction was carried out in 50-µl volume containing 50–100 ng of the total RNA, 10 µM of forward and reverse primers specific for TNF-α (GenBank accession no. M11731, forward: 5′-AGC AAA CCA CCA AGT GGA GG-3′ and reverse: 5′-CAA GGT ACA ACC CAT CCG CT-3′), 1× PCR buffer with Mg²⁺, 0.2 mM 2-deoxynucleotide 5′-triphosphate, 5 mM dithiothreitol solution, 5–10 units of RNase inhibitor, and 0.05 U/µl reaction of the enzyme mix (high-fidelity enzyme mix, RT, and AMV in storage buffer). An initial RT step was performed at 50°C for 30 min and 94°C for 2 min for one cycle, followed by 35 cycles (denaturation 94°C for 30 s, annealing at 59°C for 45 s, and extension at 68°C for 30 s), and finally, one cycle at 68°C for 7 min. In addition, a pair of primers was designed to amplify a portion of the mouse β-actin transcript that spans an exon/exon boundary (GenBank accession no. W82269, forward: 5′-CCT TCA ACA CCC AGC CAT GT-3′ and reverse: 5′-TGT GGA CCA CCA GAG CCT TCA ACA CCC AGC CAT GT-3′). β-Actin was used as a “housekeeping gene” to provide an internal marker for mRNA integrity within the experiment. PCR products were separated on (1% wt/vol) agarose gels, visualized by ethidium bromide staining under UV light. Image capture and density analysis of bands were done with the SynGene gel documentation system (SynGene-Synoptics, Cambridge, UK).

For evaluation of caspase-8 presence and activity, tissue samples were homogenized in lysis buffer. For Western blott, 25–30 µg of total protein from the tissue extract were separated on a 10% SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes (Hybond-C; Amersham Pharmacia Biotech, Piscataway, NJ) in a semidry blotting chamber. After blockage of nonspecific binding sites with 5% nonfat milk in PBS containing 0.1% Tween-20, membranes were incubated in 1:1,000 dilution of antivaspect-8 rabbit polyclonal antibody (Santa Cruz Biotechnology) for 2 h at room temperature. After extensive washing, the nitrocellulose membrane was incubated with anti-rabbit IgG conjugated with horseradish peroxidase (final concentration 1:2,000) for 90 min at room temperature. Bound antibodies were detected with enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech) according to manufacturer’s instruction. Image capturing and density analysis of bands were again performed using the SynGene gel documentation system (SynGene-Synoptics).

Caspase-8 activity was determined by a colorimetric assay (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. In brief, tissue extract of 100 to 200 µg of total protein were incubated with 5 µl of a caspase-8 specific peptide conjugated with IETD-p-nitroaniline in reaction buffer at 37°C for 2 h; then light absorption was read at a wavelength of 405 nm together with controls. Results were compared between groups.

Statistical analysis of data was performed by one-way ANOVA with Tukey’s test using a statistical software pack-

AJP-Gastrointest Liver Physiol • VOL 283 • SEPTEMBER 2002 • www.ajpgi.org
This response was diminished by anti-TNF-α. Decrease in apoptotic cell number was seen after burn. and apoptotic bodies were much more likely to be found different among groups (Fig. 4). TUNEL-positive cells number of PCNA positive stained cells per crypt was not staining, were found in the crypt. However, the num-

Values are mean mg/g body wt ± SE; n = 8 mice. aP < 0.05 vs. by ANOVA and/or Tukey’s test, TBSA, total body surface area; TNF-α, tumor necrosis factor-α.

RESULTS

Over the 12-h study period, no significant changes in body weights were seen. Total liver weights were not different between groups. Wet weights of the proximal small bowel decreased in burned and anti-TNF-α antibody-treated animals compared with controls (Table 1). Dry weights of the proximal small bowel showed a similar decrease.

Histologic measurements showed significantly decreased mucosal height after severe burn (447 ± 30 vs. 602 ± 20 μm; P < 0.05). Animals treated with anti-TNF-α showed significantly less mucosal atrophy (533 ± 12 vs. 447 ± 30 μm, P < 0.05) than untreated animals (Table 2 and Fig. 1). Treatment with neutralizing antibody alone in unburned animals did not show morphological changes compared with unburned controls. When villus heights and crypt depth were analyzed separately, only villus height was decreased by burn and again was partially restored by anti-TNF-α, whereas crypt depth was not affected (Fig. 2). A similar pattern was seen in the total mucosal cell number. In burned animals, total mucosal cell number was significantly decreased. This effect is partially, but not completely, restored by anti-TNF-α treatment (Fig. 3). Again, the main changes were seen in the villus and not in the crypt.

Proliferating intestinal cells, as identified by PCNA staining, were found in the crypt. However, the number of PCNA positive stained cells per crypt was not different among groups (Fig. 4). TUNEL-positive cells and apoptotic bodies were much more likely to be found in sections of burned animals. An over threefold increase in apoptotic cell number was seen after burn. This response was diminished by anti-TNF-α treat-

DISCUSSION

Our results show that the response of gut epithelium to severe burn is a decrease in small bowel weight, which occurs within 12 h of injury. These changes take

| Table 1. Wet and dry weight as an indicator of atrophy in proximal small bowel |

<table>
<thead>
<tr>
<th>Wet weight (mg)</th>
<th>Dry weight (mg)</th>
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<tbody>
<tr>
<td>Control</td>
<td>Control + Anti-TNF-α</td>
</tr>
<tr>
<td>21.3 ± 0.6</td>
<td>21.5 ± 0.6</td>
</tr>
<tr>
<td>12.6 ± 0.3</td>
<td>12.0 ± 0.5</td>
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Values are mean mg/g body wt ± SE; n = 8 mice. aP < 0.05 vs. control; †P < 0.05 vs. control animals (n = 8; P < 0.05). In anti-tumor necrosis factor-α (TNF-α)-treated animals (n = 8) mucosal height is significantly higher vs. burned animals (P < 0.05) but is not significantly different from control animals.

Fig. 1. Mucosal height measured on hematoxylin- and eosin-stained tissue sections. Mucosal height for animals with 30% total body surface area (TBSA) burn (n = 8) is significantly reduced compared with control animals (n = 8; P < 0.05). In anti-tumor necrosis factor-α (TNF-α)-treated animals (n = 8) mucosal height is significantly higher vs. burned animals (P < 0.05) but is not significantly different from control animals.

![Fig. 1](image_url)

| Insert Caption Here |

| Table 2. Histologic measures for atrophy in proximal small bowel |

<table>
<thead>
<tr>
<th>Mucosal height (μm)</th>
<th>Villus height (μm)</th>
<th>Crypt depth (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control + Anti-TNF-α</td>
<td>30% TBSA</td>
</tr>
<tr>
<td>602 ± 20</td>
<td>609 ± 23</td>
<td>447 ± 30*</td>
</tr>
<tr>
<td>510 ± 21</td>
<td>502 ± 22</td>
<td>345 ± 28*</td>
</tr>
<tr>
<td>113 ± 6</td>
<td>107 ± 4</td>
<td>101 ± 3</td>
</tr>
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</table>

Values are mean micrometers ± SE; n = 8 mice. aP < 0.05 vs. control; †P < 0.05 vs. control animals (n = 8; P < 0.05). In anti-tumor necrosis factor-α (TNF-α) treated animals (n = 8) villus height is significantly higher vs. burned animals (P < 0.05) but does not reach the level as in control animals (P < 0.05).

![Fig. 2](image_url)

![Fig. 3](image_url)
place without concomitant changes in total body weight. This weight loss in the proximal small bowel is an indication that injury induces gut mucosal atrophy and that this atrophy occurs very early after injury (within 12 h). Histologic changes associated with this finding are loss of mucosal height and cell number. The changes after injury occur predominantly in the villus region. In this study severe burn did not induce any changes in intestinal cell proliferation. However, intestinal cell apoptosis occurred at a threefold higher rate in burned animals. Additionally, we found that atrophic changes in mucosal height and cell mass could be partially restored by treatment with a neutralizing anti-TNF-α antibody, implicating TNF as an effector of gut mucosal changes seen after severe burn. The effect is clearly more pronounced in the villus region and does not seem to occur in the crypt. Increased apoptosis in burned animals was diminished by anti-TNF-α treatment without changes in proliferation.

As previously shown by our group, the loss of gut epithelial cells after severe burn is due to an increase in apoptotic cell death (19). This was coupled with an increase in proliferation, indicating increased cell turnover after injury. Maximum response in gut epithelium in this model was seen at 12 h after burn, after which the response quickly diminishes (19). Although similar effects may also be seen at earlier or later time points, the most prominent changes were seen at 12 h, making this a valuable time point to investigate the role of TNF-α in gut epithelial apoptosis. Potential mechanisms for these events are changes in gut perfusion with burn or the systemic effect of inflammatory mediators released immediately following injury. Studies have shown that in rat models, ischemia alone is able to induce apoptosis in jejunal and ileal mucosa, which is further exacerbated by reperfusion effects (6, 10, 17).

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**Fig. 3.** Mucosal cell number counted as cells per villus and crypt on hematoxylin- and eosin-stained tissue sections. Cell count for animals with 30% TBSA burn (n = 8) is significantly reduced compared with control animals (n = 8; P < 0.05). In anti-TNF-α treated animals (n = 8) mucosal cell count is significantly higher vs. burned animals (P < 0.05) but is not significantly different from control animals.

**Fig. 4.** Cellular proliferation determined as proliferative cell nuclear antigen (PCNA)-positive stained cells. Cell count for animals with 30% TBSA burn (n = 8) and burned anti-TNF-α treated animals shows no significant difference in numbers of PCNA-positive cells compared with control animals (n = 8 each).

**Fig. 5.** Apoptotic cell number as terminal deoxyuridine nick-end labeling (TUNEL)-positive cells or apoptotic bodies per 1,000 mucosa cells. The number of TUNEL-positive cells for animals with 30% TBSA burn (n = 8) is significantly increased compared with control animals (n = 8; P < 0.05). In anti-TNF-α treated animals (n = 8) the number of apoptotic cells is significantly decreased vs. burned animals (P < 0.05) but not significantly different from control animals.

**Fig. 6.** Local TNF-α mRNA expression by RT-PCR as ratio of TNF-α mRNA-to-β-actin mRNA shows no significant differences among groups.
However, this appears not to be the cause for the changes observed in severe burn. When addressing this issue we showed in a recent study that significant gut hypoperfusion occurs with severe burn injury within the first 4 h (14). When inducing a comparable hypoperfusion in the gut of unburned animals, the apoptotic index did not differ from normal controls, indicating that hypoperfusion induced by burns is not sufficient by itself to cause the observed increase in apoptosis. This confirms that, in fact, the atrophic changes are induced by increased apoptosis of gut epithelial cells and shifts the point of interest to other potential initiation mechanisms of apoptosis.

Several soluble and membrane-bound factors are known to induce apoptosis. Activation of members of the TNF-receptor superfamily as well as associated proteins (TNFR, TRAIL, TRADD, FADD, etc.) has been implicated as a potential mechanism in many cell systems as an inductor of apoptosis (1). Garside et al. (4) showed that a single dose of TNF-α alone is able to induce typical small bowel pathology, which is manifested as crypt hyperplasia and villus atrophy with 15 min of application. In a study by Piguet et al. (13) neutralization of TNF-α in a murine acute graft-versus-host-disease (GVHD) model reduced target organ damage. Recently, Stuber et al. (16) showed in a murine GVHD-model that FasL-Fas interaction is not involved in the induction of apoptosis of the small intestinal mucosa. The neutralization of TNF-α reduced the amount of apoptosis and the extent of mucosal atrophy. Another study by Guy-Grand et al. (5) showed increased villous cell apoptosis in TNF-α treated normal animals, which appears to be dependent on the presence of intraepithelial lymphocytes. This differs from increased apoptosis in lymphatic tissues such as spleen and thymus found in reaction to burn injury, which was linked to increased FasL mRNA expression and increased caspase-3 activity (2, 3). Apparently the apoptotic response is triggered differently in distinct tissues.

Triggering of the apoptotic response by TNF-α in gut epithelium may be initiated by either increased systemic levels acting locally in the epithelium or by increased local production of TNF-α in the gut mucosa itself. Both mechanisms are valid options and could explain the observed phenomenon of increased gut epithelial apoptosis.

Systemic levels of TNF-α have been found to be elevated after burns by several authors (7, 8, 21, 22) with burn injury alone as well as with an additional second hit. Systemic effects of TNF-α neutralization were investigated by O’Riordain et al. (12) who described a time and dose dependency of survival in a burn and septic challenge model. Efficacy of treatment depended on intrinsic TNF-α production, showing greatest effects of neutralization with maximum TNF-α levels. Applying these results to our study led us to an early application of anti-TNF-α with knowledge of sharp and transient increase in systemic TNF-α levels. However, we were not able to detect differing TNF-α levels with burn injury at the 12-h time point. This leads to the assertion that only early neutralization might be effective and, secondly, that TNF-α may only act as a transient trigger of apoptosis in the small bowel by activating a cascade of events, which in turn, consolidates the tissue response.

On the other hand Ogle et al. (11) and Wu et al. (20) showed increased local production of TNF-α in enterocytes and hepatocytes of guinea pigs after thermal injury. This increase in local expression of TNF-α in small bowel may also induce apoptotic changes. These changes in local TNF-α levels could be due to increased transcription or increased translation. However, we were not able to detect specific differences in local TNF-α mRNA expression. Here again, TNF-α may only act as a trigger mechanism for induction of apoptosis at an early time point after burn and not be expressed at elevated levels at the 12-h time point. The reported findings do exclude either of these mechanisms but also are not able to support either of these hypotheses. However, the presence of increased TNF-α levels may be irrelevant, as effects may also be realized with increased activity below the sensitivity of our methods. To elucidate the effective source of the TNF-α trigger requires further investigation.

The activation phase of cell death includes a variety of transduction pathways with signals implicating FasL-receptor interactions (FasL-Fas, TNF-α-TNFR) and the additional proteins (FADD, RIP, TRADD) (15, 18). These proteins, acting as intermediaries with “death domains,” in turn activate procaspase-8 and consequent caspase-3 and -6. Although we were not able to show differences in caspase-8 activation with anti-TNF-α treatment, this does not preclude a TNF-α-mediated mechanism for increased gut epithelial apoptosis. Because the absolute number of cells undergoing apoptosis at a certain time point is 3–8 cell per 1,000 mucosal cells, differences on the protein expression levels would be predicted to be small, if at all detectable. However, the effects at a physiological level, here as mucosal atrophy, are clearly demonstrated and suggest that TNF-α is an effector, at some level, of diminished gut mucosal integrity.
It is speculated that the administration of anti-TNF-α in the early phase of injury binds to systemic serum TNF-α or locally abundant TNF-α. The early neutralization of circulating soluble and transmembrane forms of TNF-α thus removes the initiating factor for the apoptotic cascade in gut mucosa. A prevention of TNF-α/TNFRI interaction would reduce effective signaling with caspase activation. An alternative mechanism might be the inhibition of signals activating the mitogen-activated protein kinase pathway (9).

From this study, we conclude that changes of gut mucosal homeostasis seen after severe burn are associated with activation of apoptosis by TNF-α/TNFRI interaction. The effect of TNF-α neutralization on gut mucosal homeostasis partially reversed mucosal atrophy after burns in this study. It remains open for discussion whether this is, in fact, beneficial on the gut or systemic level. Anti-TNF-α strategies were indeed successful to improve survival and outcomes in injured animals (12). However, similar use in patients was unsuccessful. Only in defined studies in other animal models and perhaps in patients will it be possible to determine whether TNF-α neutralization may be of benefit. Whether these findings can be used to clinical advantage will require further study to elucidate the source of TNF-α involved in the response and, secondly, to determine whether inhibiting mucosal atrophy after burn is of clinical benefit.

This study was supported by Shriners Hospitals for Children Grant 8580. This study was presented, in part, at the Surgical Forum of the Annual Meeting of the American College of Surgeons, Chicago, IL, October 22–28, 2000.

REFERENCES