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Apoloptosis: One of the Mechanisms That Maintains Unresponsiveness of the Intestinal Mucosal Immune System

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Intestinal mucosa is constantly exposed to environmental Ags. Activation of lamina propria (LP) T cells by luminal Ags may lead to the production of inflammatory cytokines and subsequent mucosal inflammation and tissue damage. However, in normal circumstances, LP T cells do not respond to antigenic stimulation. The mechanisms of this unresponsiveness in healthy subjects are not fully understood. In this study, we found by in vivo analysis that, except for T cells in lymph nodules of the mucosa, 15% of LP T cells underwent apoptosis in normal individuals. In contrast, there was a marked reduction in apoptosis of LP T cells in patients with inflammatory bowel disease (Crohn’s disease and ulcerative colitis) and those with specific colitis. Our findings suggest that apoptosis might be a mechanism that turns off mucosal T cell responses to environmental Ags in healthy subjects, and resistance to apoptosis could be an important cause of mucosal immune dysregulation and tissue inflammation in colitis. The Journal of Immunology, 2001, 166: 6399–6403.

Although mucosal surfaces are exposed to many environmental Ags, the host’s immune system remains unresponsive to normal luminal Ags. The mechanisms underlying that phenomenon are only partially understood. Oral administration with soluble proteins in mice induces tolerance by inducing regulatory T cells in mucosal organized lymphoid tissues (1) and by anergizing or deleting Ag-specific T cells (2–6). In humans lamina propria (LP)4 T cells in normal individuals do not proliferate or produce T cell cytokines such as IL-2 and IFN-γ in response to TCR stimulation (7–10). However, T cells do proliferate in mucosal organized lymphoid tissue such as Peyer’s patch, as determined in situ (10), suggesting that there are local environmental differences for T cell activation in organized lymphoid tissue and in diffuse lymphoid tissue such as LP. The Ag-specific response of LP T cells is down-regulated as a result of impaired signal transduction through the TCR-CD3 complex (7, 8, 11, 12). The reduced response of LP T cells in normal individuals might be induced by locally produced, small, nonprotein molecules with oxidative properties (13). Furthermore, LP macrophages do not provide costimulation to T cells for proliferation in response to TCR stimulation (14). In inflamed intestinal mucosa, as in Crohn’s disease, increased numbers of proliferating LP T cells have been documented (10, 15); proinflammatory cytokines, such as IL-1β, IL-6, IL-15, IFN-γ, and TNF-α, that are not expressed in normal mucosa are produced in the inflamed mucosa (10, 16), suggesting that LP T cells and macrophages might have been activated as a result of the loss of normal regulatory mechanisms.

Cell death by apoptosis regulates the lymphocyte population and terminates immune responses at sites of Ag exposure. Because LP is constantly exposed to environmental Ags, it is highly possible that lymphoid cells in LP are also regulated by apoptosis, i.e., apoptosis might prevent the activation and clonal expansion of lymphocytes to unharmful environmental Ags. It has been shown that isolated human LP T cells have a tendency to undergo apoptosis in vitro (17–19). In vitro, upon stimulation of LP T cells with anti-CD2 Ab, the cells underwent apoptosis, which was mediated via the CD95 pathway (17). Interestingly, CD2-mediated apoptosis is reduced in patients with Crohn’s disease (19), and the resistance of T cells to apoptotic signals is associated with a higher ratio of Bel-2 to Bax (20). However, no study has yet shown that LP T cells undergo apoptosis in vivo to confirm the physiologic significance of mucosal T cell apoptosis. In this study we determined whether LP lymphocytes (T cells and plasma cells) underwent apoptosis in situ in normal mucosa. As a comparison we also determined the apoptotic status of those cells in the inflamed mucosa of patients with colitis where lymphoid cells should have a higher survival rate. We found that a significant amount of LP lymphocytes underwent apoptosis in normal mucosa and that apoptosis was greatly reduced in mucosal inflammation.

Materials and Methods

Subjects

All subjects had endoscopic examination as part of their clinical evaluation. All patients with colitis had an established diagnosis of ulcerative colitis (UC), Crohn’s disease (CD), or specific colitis (infectious colitis, ischemic colitis, and radiation colitis). The diagnosis of colitis was based on a standard clinical, endoscopic, and histological criteria. All patients with colitis were symptomatic and had endoscopically and histologically active disease. Symptoms included abdominal pain (n = 6), diarrhea (n = 15), hematochezia (n = 10), and urgency (n = 8). Normal individuals (controls) had screening endoscopic procedure for colon cancer. None had gastrointestinal symptoms, and all had normal endoscopic findings. Two pieces of mucosa (~8 mg) were taken from areas of active inflammation (in colitis patients) or normal-appearing mucosa (in controls) in the sigmoid colon.

Abbreviations used in this paper: LP, lamina propria; AMCA, 7-amino-4-methyl-coumarin-3-acetic acid; CD, Crohn’s disease; DAB, 3,3-diaminobenzidine; IBD, inflammatory bowel disease; UC, ulcerative colitis.

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Large fresh colonic specimens from normal mucosa were also obtained from patients who were having colon cancer surgically removed. The mucosal tissues used were taken far away from the tumor and close to the resection margin and were macroscopically and microscopically normal. The biopsies and part of surgical specimens were immediately snap-frozen in liquid nitrogen and stored at −80°C, and parts of the surgical specimens were fixed in ice-cold methanol/PBS solution before paraffin embedding for subsequent analysis. This study was approved by the institutional review board for safety of human subjects of Loyola University Stritch School of Medicine and Rush University.

**Determination of T cell apoptosis in situ**

**Detection of apoptotic cells in paraffin-embedded vs cryopreserved human colonic mucosa.** Paraffin sections (5 μm) were deparaffinized in two changes of xylene for 5 min each. Sections were hydrated in an alcohol gradient twice (100, 90, 75, and 50% for 3 min each) and incubated for 5 min in tap water. Endogenous peroxidase was quenched by incubating sections in 0.3% H₂O₂ in PBS for 10 min. Sections were washed twice for 5 min each time in PBS and incubated in a humidified chamber for 1 h at 37°C with TdT, 0.75 U/μl TdT (Life Technologies), 1× TdT reaction buffer, and 100 μM biotin-labeled dCTP (Life Technologies) for 1 h at 37°C in humidified chambers. For negative controls, human colonic sections were incubated with a similar solution without the TdT enzyme. To detect fragmented DNA with 3′ ends labeled with biotinylated-dCTP, the sections were incubated for 60 min at room temperature with 40 μg/ml streptavidin-7-amino-4-methylcoumarin-3-acetic acid (AMCA; Roche, Carpenteria, CA) in 4× SSC staining buffer containing 0.1% Nonidet P-40 (v/v; Sigma) and 5% nonfat dry milk. The sections were washed three times in cold PBS at 4°C between each incubation step. After incubation with streptavidin-AMCA solution, the sections were incubated with mouse-anti-human CD3, CD38, or CD68 mAbs and washed again. The frozen sections were then treated exactly as described for the paraffin-embedded tissues starting with the TdT incubation before being evaluated by light microscopy.

**Quantitation of apoptotic cells**

At least two biopsies from each patient were used, and all of them were examined for the presence of apoptosis. The whole tissues of each biopsy were examined. To quantify the number of apoptotic cells, after preview of the section, different fields were photographed randomly, and the total number of CD3⁺, CD38⁺, or CD68⁻ cells and respective apoptotic cells were counted by two investigators blinded to the sample groups. Four to 11 fields/tissue were counted for each subject. Apoptosis was expressed as the mean percentage of apoptotic CD3⁺, CD38⁺, or CD68⁻ cells among all

**FIGURE 1.** Detection of apoptotic cells in cryopreserved or Formalin-fixed and paraffin-embedded human colonic tissues. Frozen tissues (a and c) and Formalin-fixed, paraffin-embedded tissues (b and d) were treated with TdT and biotin-dCTP, developed with DAB, and counterstained with methyl green. Negative controls (c and d) were incubated in solution lacking TdT. Original magnification, ×400.

**FIGURE 2.** Detection of ssDNA in cells from normal human colonic tissue. Sections were stained using a mAb against ssDNA followed by a biotinylated anti-mouse secondary Ab. The sections were incubated with Extravidin-peroxidase and developed with DAB and counterstained. Brown cells in the lamina propria (a) are positive for ssDNA (arrow). As a negative control (b) an isotype control Ab or PBS was substituted for anti-ssDNA or sections were treated with S1 nuclease before application of primary Ab. Original magnification, ×400.
CD3⁺, CD38⁺, or CD68⁺ cells from all counted fields. Because of variable background staining at the margins of tissues, counting was considered unreliable in those areas, and they were excluded from the analysis.

**Detecting ssDNA in human colonic mucosa**

We followed the procedure exactly as specified in the protocol accompanying Ab to ssDNA (Alexis Biochemicals, San Diego, CA). Briefly, mucosa was removed from resected colon of cancer patients and immediately fixed in ice-cold methanol/PBS (6/1) solution. The tissue and solution were returned to −20°C for 2 days. The fixed tissue was dehydrated in two changes of absolute methanol and two changes of xylene and then embedded in paraffin. Fresh 4-μm sections were prepared before staining and heated at 56°C for 1–2 h. The sections were deparaffinized in two changes of Safeclear (Fisher, Hanover Park, IL) and incubated in three changes of methanol/PBS (6/1) solution for 20 min each. Then they were rinsed with Dulbecco’s PBS (the only PBS used throughout the procedure) and incubated for 5 min in PBS supplemented with 0.2% Triton X-100 and 5 mM MgCl₂. Sections were then heated for exactly 6.5 min in a 99°C water bath followed by placement in ice-cold PBS for 10 min. As a negative control, sections were incubated with 100 U/ml S1 nuclease (Sigma) in acetate buffer after ice-cold PBS wash, or an isotype control Ab (mouse IgM) or PBS was substituted for the anti-ssDNA mAb. Endogenous peroxidase was blocked using 3% H₂O₂ in PBS, and slides were treated with 0.1% BSA for 30 min. After rinsing, anti-ssDNA mAb was applied to sections for 15 min and washed, and biotin-conjugated rat anti-mouse IgM (Zymed, San Francisco, CA) was applied for another 15 min. After washing, ExtrAvidin-peroxidase (Sigma) was applied for 15 min. Sections were washed and developed with DAB (Sigma).

**Results and Discussion**

To determine whether LP lymphocytes undergo apoptosis in situ in normal intestinal mucosa, we initially used a TdT end-labeling assay to detect apoptotic cells in mucosal biopsies from normal individuals. Many apoptotic cells were found in normal mucosal tissues. Considering that this much apoptosis has not been reported in the literature, we determined whether our observation might be due to the methods used. Previous studies that determined the apoptosis of mucosal cells used formalin-fixed, paraffin-embedded
mucosal tissues. Therefore, we compared frozen tissues with Formalin-fixed, paraffin-embedded tissues. We obtained normal mucosal tissues. One part was snap frozen, and another part was fixed with Formalin and embedded in paraffin. Apoptosis was determined in both parts by TdT end-labeling assay. As shown in Fig. 1, more apoptotic cells in LP were detected in snap-frozen tissue than in formalin-fixed and paraffin-embedded tissue from the same subject. To further confirm the cell apoptosis in the mucosa, we used an assay to determine ssDNA, which has been shown to be a specific method for detection of apoptotic cells (22, 23). As shown in Fig. 2, ssDNA-positive cells were found in LP of normal mucosa, thus confirming that LP cells underwent apoptosis in vivo.

To determine which cells underwent apoptosis in LP, we combined indirect immunofluorescence with a TdT end-labeling assay. Apoptotic cells were stained for T cell markers (CD3), plasma cell marker (CD38), and macrophage marker (CD68). In the LP from control subjects (n = 7), a mean of 15% of LP T cells underwent apoptosis (Figs. 3 and 4). Both CD4^+ and CD8^+ T cells underwent apoptosis (data not shown). The apoptosis of LP T cells was found in all seven of the healthy control subjects. In contrast, apoptotic T cells were not found in solitary lymph nodules of control subjects (data not shown). Similarly, a mean of 20% of CD38^+ cells and 16% of CD68^+ cells were positive in the TdT end-labeling assay (Fig. 3). We used CD38 as a marker for plasma cells because of the high background when we initially used anti-human IgA and IgG to detect plasma cells. Although CD38^+ cells are most likely plasma cells, CD38 can also be expressed by activated T cells. Therefore, the percentage of plasma cells might be an overestimation. We found that CD68^+ cells were positive in the TdT end-labeling assay, possibly because macrophages are deleted to control the immune responses to normal Ags, as macrophages are potentially important APCs. However, we could not exclude the possibility that those macrophages were positive in the TdT end-labeling assay because they engulfed the apoptotic bodies/cells. That possibility will be investigated in future studies. It should also be noted that the apoptotic cells may not be evenly distributed along the colon length. Therefore, the mean percentages of apoptotic cells are just an estimate and are subject to sample error. Nonetheless, the data clearly showed that a significant proportion of T cells and plasma cells underwent apoptosis in vivo in LP of normal colonic mucosa.

It has been shown that mucosal T cells have a tendency to undergo apoptosis in vitro (17). Here we demonstrated that LP T cells, but not T cells in mucosal organized lymphoid tissue, underwent apoptosis in vivo. This finding suggests that T cells in the organized lymphoid tissue are able to respond to antigenic stimulation. Our findings are consistent with previous studies in mice showing that T cells in Peyer’s patches can proliferate and produce cytokines upon Ag stimulation (24, 25). There are at least two possible explanations for our observed difference in T cells from LP and the organized lymphoid tissues. The first possibility is that T cells that are activated in the organized lymphoid tissue preferentially home to LP where the T cells encounter normal environmental Ags and undergo apoptosis upon stimulation with an overwhelming Ag load. The second possibility is that the LP is an environment where reactive T cells are induced to undergo apoptosis so that they are not able to respond to antigenic stimulation (environment-driven apoptosis). Similarly, the apoptosis of plasma cells could be explained by two possibilities, i.e., Ag or environment-driven. To directly address whether apoptosis is caused by antigenic stimulation, an animal model is needed; however, the apoptosis of LP lymphoid cells has been detected only in humans, not in mice or rabbits (L. Qiao, unpublished observation).

We hypothesize that if apoptosis is important in controlling the responses of mucosal lymphoid cells to normal luminal Ags, then the apoptosis would be reduced in an inflammatory microenvironment where, in most cases, lymphocytes are activated. To test this hypothesis, we determined apoptosis of lymphoid cells in the patients with colitis. Apoptosis of T cells, plasma cells, and macrophages in LP was markedly reduced in all tissue samples from the patients with colitis (Figs. 3 and 4). This marked decrease in apoptosis did not appear to be due to anti-inflammatory medication, because all patients had reduced apoptosis regardless of type of therapy. In addition, there was no correlation between disease activity or disease extent and degree of apoptosis because most diseased mucosa had very low levels of T cell apoptosis. Our data also showed that reduced T cell apoptosis was not specific for UC, CD, or specific colitis and was seen in the inflamed colon regardless of the etiology. Hence, reduced apoptosis may be a factor in the immune dysregulation seen in IBD, but it may not play a primary etiological role. The data from these patients contrasted with results from murine colitis models, where increased T cell apoptosis was found in inflamed mucosa (26, 27). This finding might be due to the fact that there are few apoptotic LP T cells in normal mouse mucosa.

The apoptosis of LP T cells could occur through CD95 and/or other receptors. It has been shown that most LP T cells isolated from uninflamed areas of resected colon carcinoma expressed CD95, as analyzed by flow cytometry (18). When LP cells were stimulated by CD2, the T cells also underwent apoptosis, which could be blocked by anti-CD95 Ab (17). However, unstimulated LP T cells underwent apoptosis in vitro, which could not be blocked by an inhibitory anti-CD95 Ab (17). Thus, the apoptosis of unstimulated LP T cells might be induced by an uncharacterized pathway, as shown in the in vitro experiment (17). It also has been shown that T cells isolated from areas of inflammation in CD, UC, and other inflammatory states were resistant to CD2-induced apoptosis, and that resistance was accompanied by elevated Bcl-2 levels (19). T cells grown from CD lesions were resistant to CD95- or NO-mediated apoptosis, with similarly increased Bcl-2:Bax ratios (20). Those data suggest that CD95 plays a role in inducing apoptosis in LP T cells; however, other receptors or pathways by which apoptosis is induced also might be involved.

It appears that the reduced apoptosis in IBD is a consequence of inflammation rather than a cause of IBD, since it was also noted in specific colitis. Thus, reduced apoptosis could be due to proinflammatory cytokines that are abundantly present in the inflamed

FIGURE 4. Percentage of lamina propria apoptotic CD3^+, CD38^+, and CD68^+ cells in mucosa from normal individuals (norm) and from patients with UC, CD, and specific colitis (SC). The bars represent SDs of the mean percentage of apoptosis in each group of patients (the number of patients per group is shown on the top of bars). The mean percentage of apoptosis in control patients (norm) is significantly different from that of the other three groups, with p = 0.0018 for CD3^+ cells and p = 0.0001 for CD38^+ cells and for CD68^+ cells. Differences among the groups were compared using ANOVA. Duncan’s multiple range test was used for pairwise comparisons to maintain the overall a level at 0.05.
mucosa. Many proinflammatory cytokines, such as IL-1, IL-6, IL-2, IFN-γ, and IL-15, are expressed in IBD (16, 28). In particular, IL-15 might have an important effect in counteracting apoptosis of LP cells, because it was shown that IL-15 was produced by macrophages in inflamed mucosa of patients with IBD (28). Bel-2 expression can be induced as a result of signaling via the common γ-chain of the IL-2 receptor, which serves as a signaling component of the receptor for IL-2, IL-4, IL-7, and IL-15. Thus, IL-15 might greatly enhance the expression of Bel-2, which further enhances resistance of LP T cells against induction of apoptosis.

In summary, we demonstrated that LP T cells underwent apoptosis in normal mucosa, which might have important effects in maintaining the unresponsiveness of LP T cells to normal luminal Ags and the homeostasis of mucosal lymphoid tissue. The level of LP-cell apoptosis is greatly reduced in the inflamed colonic mucosa, which may contribute to the mucosal immune dysregulation noted in IBD.

Acknowledgments

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