

# Epithelial apoptosis in mechanistically distinct methods of injury in the murine small intestine

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**Summary.** Gut epithelial apoptosis is involved in the pathophysiology of multiple diseases. This study characterized intestinal apoptosis in three mechanistically distinct injuries with different kinetics of cell death. FVB/N mice were subjected to gamma radiation, *Pseudomonas aeruginosa* pneumonia or injection of monoclonal anti-CD3 antibody and sacrificed 4, 12, or 24 hours post-injury (n=10/time point). Apoptosis was quantified in the jejunum by hematoxylin and eosin (H&E), active caspase-3, terminal deoxynucleotidyl transferase dUTP-mediated nick end labeling (TUNEL), in situ oligoligation reaction (ISOL,) cytokeratin 18, and annexin V staining. Reproducible results were obtained only for H&E, active caspase-3, TUNEL and ISOL, which were quantified and compared against each other for each injury at each time point. Kinetics of injury were different with early apoptosis highest following radiation, late apoptosis highest following anti CD3, and more consistent levels following pneumonia. ISOL was the most consistent stain and was always statistically indistinguishable from at least 2 stains. In contrast, active caspase-3 demonstrated lower levels of apoptosis, while the TUNEL assay had higher levels of apoptosis in the most severely injured intestine regardless of mechanism of injury. H&E was a statistical outlier more commonly than any other stain. This suggests that regardless of mechanism or kinetics of injury, ISOL correlates to other quantification methods of detecting gut epithelial apoptosis more than any other method studied and compares favorably to other commonly accepted techniques of quantifying apoptosis in a large intestinal cross sectional by balancing sensitivity and specificity across a range of times and levels of death.

**Key words:** Apoptosis, Intestine, Radiation, Sepsis, Anti CD3

## Introduction

Apoptosis is an evolutionary conserved, ubiquitous method of programmed cell death required for the function of multicell organisms (Kerr et al., 1972). While apoptosis is necessary for both development and homeostasis, perturbations in apoptotic pathways have been directly linked to the pathophysiology of multiple disease processes, and manipulating apoptosis has been proposed to have substantial potential for therapeutic gain (Reed, 2002; Fischer et al., 2005; Garber, 2005).

The intestine is unique among solid organs because of its high cell turnover, with epithelial proliferation, differentiation and cell elimination occurring within a few days (Cheng and Leblond, 1974; Cheng and Bjerkenes, 1982). Although cells can be removed from the intestinal crypts and villi by either apoptosis or exfoliation into the lumen, mathematical models suggest that apoptosis accounts for the bulk of cell loss in the intestine (Hall et al., 1994). Both increased and decreased gut epithelial apoptosis have been implicated in the pathophysiology of multiple local and systemic disease states including (but not limited to) sepsis, cancer, radiation-injury, inflammatory bowel disease, ischemia/reperfusion, burn injury, and necrotizing enterocolitis (Clarke et al., 1994; Merritt et al., 1994; Coopersmith et al., 1997; Noda et al., 1998; Wolf et al., 1999; Ramachandran et al., 2000; Watson and Pritchard, 2000; Marshman et al., 2001; Coopersmith et al., 2002a,b; Houchen et al., 2003; Zeissig et al., 2004; An et al., 2005; Bowen et al., 2005; Mao et al., 2005; Sanders, 2005; Zhou et al., 2005). Depending on the inciting event, gut epithelial apoptosis can occur via the receptor-mediated (extrinsic) pathway (Strater and Moller, 2003; Riehl et al., 2004), the mitochondrial (intrinsic) pathway (Knott et al., 2003), or a combination of both pathways (Tang et al., 2004). The kinetics of gut epithelial apoptosis vary widely depending on what initiates cell death, with the onset of death occurring within a few hours or greater than a day following the inciting event.

Unfortunately, there is no gold standard for

identifying apoptosis in a broad cross section of gut epithelium (Groos et al., 2003). Transmission electron microscopy is extremely accurate at identifying apoptosis on small pieces of tissue, but is not quantifiable or feasible for examining apoptosis across multiple crypt/villus units. Since gut epithelial apoptosis can be patchy in certain disease processes (sepsis is one example), the ability to identify and quantify death on a broader scale is best carried out using immunohistochemistry. Generally, identification of apoptotic cells can be broken down into morphologic and functional criteria. Gut epithelial apoptosis can be readily quantified morphologically using H&E staining. However, this has the obvious limitation that morphologic changes must be readily visible in order to identify them on H&E which means a) this is not useful in the early stages of death when a cell has committed to die but gross evidence is not yet present, b) it may be difficult to distinguish from necrosis in later stages of apoptosis, and c) quantification is subject to significant inter-observer variability (Darzynkiewicz et al., 2001). Multiple functional stains have been used in quantifying gut epithelial apoptosis including TUNEL (which was initially described in the intestine (Gavrieli et al., 1992)), ISOL, active caspase-3, M30 (the caspase cleaved product of cytokeratin 18), and annexin V. Taken as a whole, these stains have the ability to identify both early (annexin V) and late (TUNEL, ISOL, active caspase-3) apoptosis. However, although a large literature exists on how to identify apoptosis, this is frequently species-specific, tissue-specific, injury-specific, mechanism-specific, and time-specific, and frequently questions arise as to the accuracy of the staining method being studied (Wolvekamp et al., 1998; Walker and Quirke, 2001).

In this study, we examined multiple techniques of quantifying gut epithelial apoptosis in three injuries – gamma radiation, *P. aeruginosa* pneumonia, and monoclonal anti CD3 injection. Each induces gut epithelial apoptosis through unique mechanisms and pathways, and each induces cell death with unique kinetics, with apoptosis appearing and peaking at distinct time points. A comparison of immunohistochemical techniques across a spectrum of inciting injuries, cellular pathways, and times following injury onset was undertaken to determine if a common approach could be identified for identifying gut epithelial apoptosis, regardless of the clinical scenario.

## Materials and methods

### Animals

Experiments were performed on six to eight week old male FVB/N mice. Experimental animals were sacrificed at 4, 12, or 24 hours after injury (10 mice/time point/injury). All injuries were performed at a similar time of day (midmorning) to minimize diurnal variation. Sham operated animals (n=4) was also sacrificed 24

hours following intratracheal injection of 0.9% NaCl. These animals appeared healthy at time of sacrifice and were indistinguishable from unmanipulated animals when stained for apoptosis (data not shown). Animals were maintained on 12 hour light-dark cycles with free access to food and water at all times. All experiments were conducted in accordance with the National Institutes of Health guidelines for the use of laboratory animals and were approved by the Washington University Animal Studies Committee.

### Injury models

#### Gamma radiation

Gamma radiation induces gut epithelial apoptosis via a p53-dependent (Clarke et al., 1994; Merritt et al., 1994), TNFR1-dependent (Riehl et al., 2004) mechanism that is also partially dependent on IGF-1 (Wilkins et al., 2002) and Bcl-2 (Coopersmith et al., 1999). Whole-body irradiation of mice was carried out in a Gammacell 40 137Cs irradiator (Atomic Energy of Canada Ltd.) at a dose rate of 77.6 cGy/min and a total dose of 6 Gy.

#### *P. aeruginosa* pneumonia

*P. aeruginosa* pneumonia induces gut epithelial apoptosis via the mitochondrial pathway via a Bcl-2 dependent mechanism (Coopersmith et al., 2002b, 2003). Under halothane anesthesia, a midline cervical incision was made and each animal received an intratracheal injection of 40  $\mu$ l of a solution containing the ATCC 27853 strain of *P. aeruginosa*. Following this, animals were held upright for 10 seconds to enhance delivery of bacteria into the lungs, and the incision was closed in two layers. The final density of the inoculum was between  $5 \times 10^8$  and  $1 \times 10^9$  colony-forming units/milliliter as determined by serial dilution and colony counts. Sham mice were handled identically, and received an equivalent volume of 0.9% NaCl without bacteria.

#### Anti CD3

Anti CD3 induces gut epithelial apoptosis via a TNF/Fas-dependent, Bcl-2 independent pathway (unpublished observations) although published studies indicate that cell death is not increased in the first 3 hours after antibody injection (Clayburgh et al., 2005). Hamster anti-mouse CD3 monoclonal antibody (2C11) was purified from hybridoma supernatant (Musch et al., 2002). Animals were given 200 mcg of anti CD3 antibody via intraperitoneal injection.

### Apoptosis quantification

Immediately following sacrifice, the entire small intestine was removed and the middle 4 centimeters

## Apoptosis in the murine small intestine

(distal jejunum) was isolated. This segment of intestine was then opened along the cephalocaudal axis, washed in 10% buffered formalin to remove luminal contents and then fixed in formalin for 24 hours. An identical length at the same portion of the intestine was taken in all animals to exclude the effects of geographic variability on intestinal apoptosis.

Apoptosis was quantified in 100 crypts from well-oriented crypt-villus units, defined as a crypt sectioned parallel to the crypt-villus axis with Paneth cells at the crypt base and an unbroken epithelial column extending to the villus tip (Coopersmith et al., 2002b; Husain et al., 2005; Javadi et al., 2005). Each stain was quantitated on two noncontiguous tissue sections for a total of 200 crypts per animal. The data presented for each time point on each injury represents the quantitation of 20 slides (2 slides on 10 mice).

### Hematoxylin and Eosin (H&E) staining

Apoptotic cells were identified by characteristic morphology of nuclear fragmentation (karyorrhexis) and cell shrinkage with condensed nuclei (pyknosis).

### Active caspase-3 staining

Following dewaxing and rehydration of paraffin-embedded sections, endogenous peroxidase activity was blocked by incubating in 3% H<sub>2</sub>O<sub>2</sub> in methanol at 23°C for 15 minutes. To facilitate antigen retrieval, sections were then microwaved in citrate buffer (pH 6.0) for 9 minutes, followed by the application of polyclonal rabbit anti-active caspase-3 (1:100 in PBS, Cell Signaling Technology Inc, Beverly, MA), for 60 minutes at 23°C. This was followed by a secondary biotinylated goat anti-rabbit antibody for 30 minutes (1:200, Vector Laboratories, Burlingame, CA). Slides were then incubated with VECTASTAIN ABC (Vector Laboratories), developed with DAB, and counterstained with hematoxylin.

### TUNEL assay

The TUNEL assay was performed using a commercially available kit according to manufacturer specifications (Roche Diagnostics, Indianapolis, IN) (Lesauskaite et al., 2004). Of note, previous data from our laboratory has shown that multiple commercially available TUNEL kits yield similar results, and this kit was chosen based upon published literature. After rehydration, slides were permeabilized with Proteinase K (20 µg/mL, SIGMA, St Louis) for 23°C and then rinsed 4 additional times with distilled water followed by incubation with a solution made up of terminal deoxynucleotidyl transferase (TdT; 1 µL/200 µL of mix solution), bovine serum albumin (1 mg/mL), and biotin-16-dUTP (1 nmol/50 µL of mix solution) in TdT buffer. Sections were then incubated in saline citrate for 15 minutes, rinsed, and incubated with 2% bovine serum albumin at 23°C. Endogenous peroxidase activity was

then blocked via an incubation with 3% H<sub>2</sub>O<sub>2</sub>. The labeled DNA fragments were detected with peroxidase-conjugated antibody elicited against biotin. Slides were then developed with DAB, and counterstained with hematoxylin.

### ISOL

ISOL was performed using a commercially available kit according to the manufacturer instructions (Apoptag, Chemicon International, Temecula, CA). Sections were rehydrated and permeabilized similar to above except incubation with Proteinase K was performed at a concentration of 50 µg/ml (Lesauskaite et al., 2004). After a brief (10 seconds) incubation with equilibration buffer, slides were incubated with T4 DNA ligase enzyme and oligo A (1:9 µL) in a humidified chamber for 16 hours. Slides were then washed and incubated with streptavidin-peroxidase conjugate. Slides were developed with DAB and counterstained with hematoxylin.

### Cytokeratin 18

Staining for the neo-epitope formed after caspase cleavage of cytokeratin was performed using a commercially available kit (M-30 CytoDeath Biotin, Peviva, Bromma, Sweden). After blocking endogenous peroxidase by incubating sections in H<sub>2</sub>O<sub>2</sub>, sections were placed in heated (90°C) citric acid buffer for 20 minutes for antigen retrieval. Sections were incubated with monoclonal antibody (1:50) for 45 minutes at 23°C. After rinsing three times in PBS, sections were incubated with Avidin-Peroxidase for 1 hour at 23°C (Vectastain ABC KIT, Vector Laboratories, Burlingame, CA) rinsed, incubated for 4 minutes with DAB, rinsed and dehydrated.

### Statistics

Data were compared using one way analysis of variance followed by the Tukey post-test. Data were analyzed using the statistical program Prism 4.0 (GraphPad Software, San Diego, CA) and are presented as mean ± SEM. P values <0.05 were considered statistically significant.

### Results

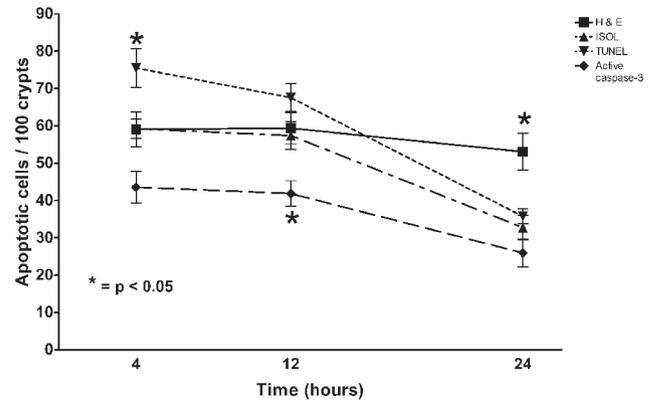
Quantifiable results were obtained for all injuries using H&E, TUNEL, ISOL, and active caspase-3 staining. While levels of gut epithelial apoptosis varied depending on the injury studied (detailed below), levels of apoptosis in either unmanipulated or sham-operated animals were similar for each of these stains, ranging from 3.3±0.5 apoptotic cells/100 crypts for active caspase-3 to 4.5±0.5 apoptotic cells/100 crypts for H&E.

Sporadic epithelial cells stained for cytokeratin 18 but at a much lower level than the other stains (data not shown). Since the consistent increases seen in gut

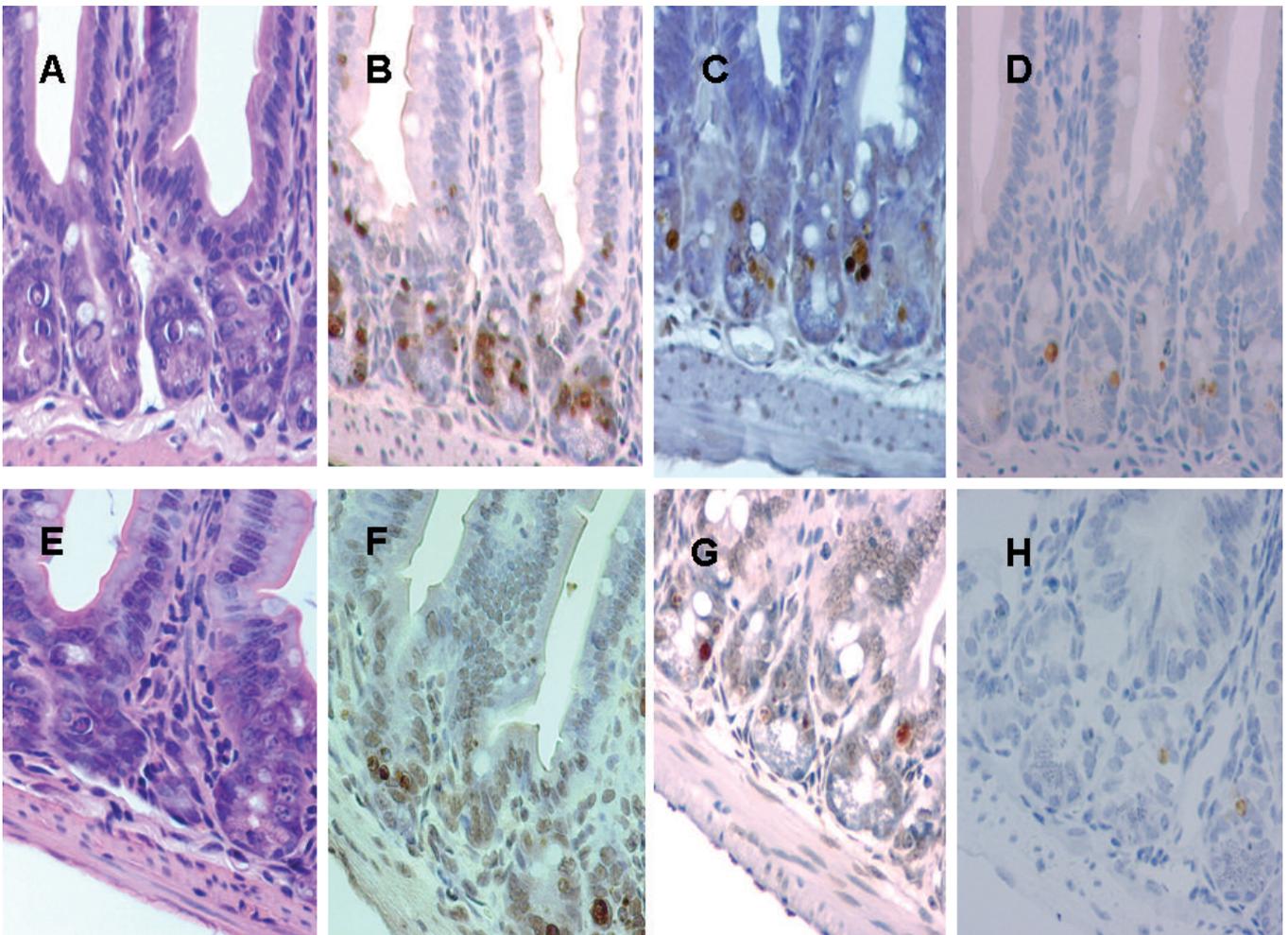
epithelial apoptosis seen with each injury were not identified using cytokeratin 18 staining, this was not quantified. Despite using published protocols (Bronckers et al., 2000; Paris et al., 2001; Bodiga et al., 2005) and greater than 15 additional modifications, reproducible annexin V staining could not be obtained (data not shown).

#### Gamma radiation

Apoptosis levels were elevated by 4 hours following the onset of ionizing radiation and remained above levels seen in unmanipulated mice at both 12 and 24 hours (Figs. 1, 2). Depending on the time point examined, levels of apoptosis varied by as much as two fold with  $53.0 \pm 4.9$  apoptotic cells/100 crypts for H&E 24 hours after radiation compared to  $25.9 \pm 3.7$  apoptotic cells/100 crypts for active caspase-3 ( $p < 0.001$ ). A different stain was statistically different from the remaining three stains

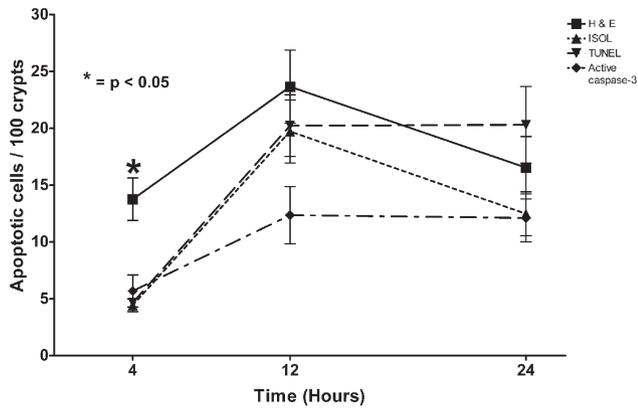


**Fig. 1.** Quantification of apoptosis in mice receiving Gamma radiation. H&E, TUNEL, ISOL and active caspase-3 are shown at 4, 12, and 24 hours. Asterisk represents a value statistically different from the other three values.

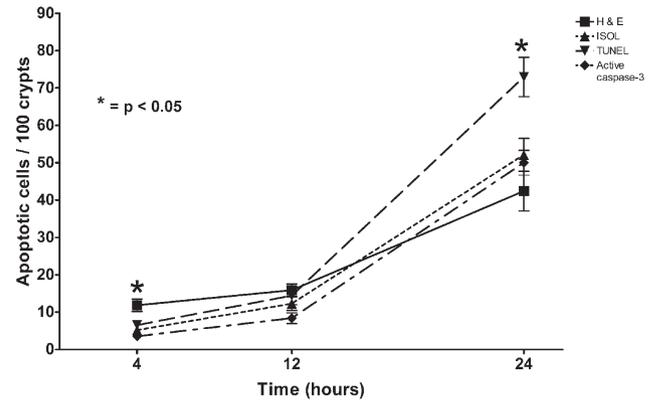


**Fig. 2.** Apoptosis in gut epithelial tissue from mouse that received gamma radiation 4 (A-D) or 24 (E-H) hours earlier. Sections are stained for H&E (A, E), TUNEL assay (B, F), ISOL (C, G), and active caspase-3 (D, H). Apoptosis is easily identifiable by morphology or by brown staining. While apoptosis is markedly elevated in each section, there are less apoptotic cells/crypt when stained for active caspase-3.

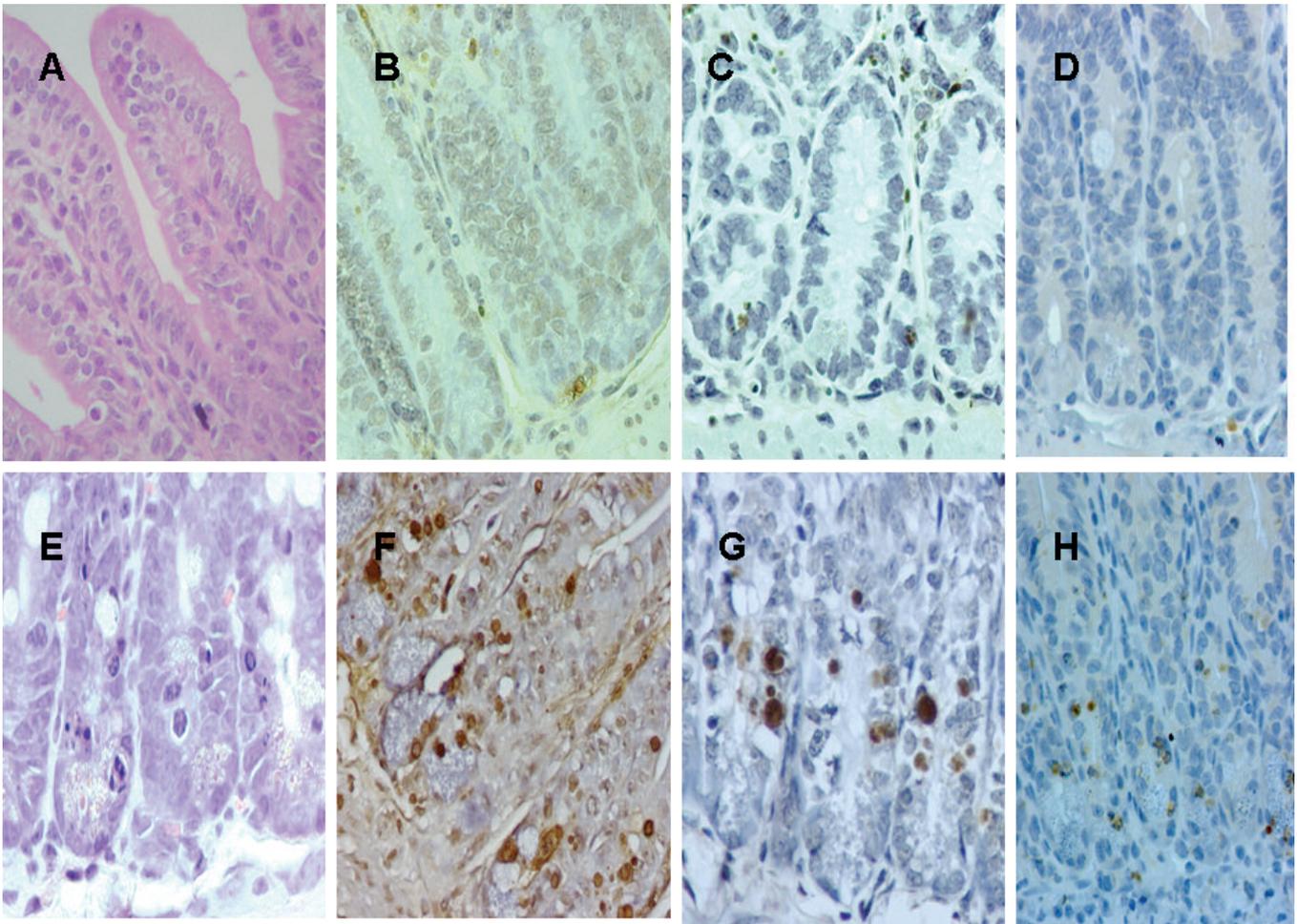
### Apoptosis in the murine small intestine



**Fig. 3.** Quantification of apoptosis in mice receiving *P. aeruginosa* pneumonia. Asterisk represents a value statistically different from the other three values.



**Fig. 4.** Quantification of apoptosis in mice receiving anti CD3. Asterisk represents a value statistically different from the other three values



**Fig. 5.** Apoptosis in gut epithelial tissue from mouse that received anti CD3 4 (A-D) or 24 (E-H) hours earlier. Sections are stained for H&E (A, E), TUNEL assay (B, F), ISOL (C, G), and active caspase-3 (D, H). Apoptosis is easily identifiable by morphology or by brown staining. While apoptosis is markedly elevated in each section, there are more apoptotic cells/crypt with the TUNEL assay.

at each time point – TUNEL was higher than the other stains at 4 hours, active caspase-3 was lower than the other stains at 12 hours, and H&E was higher than the other stains at 24 hours (all  $p < 0.05$  compared to all other stains at a specific time point). Although the stains that were similar varied depending on time point, ISOL was never a statistical outlier.

#### *P. aeruginosa pneumonia*

Although peak apoptosis levels were lower in pneumonia than either of the other two injuries studied, intestinal apoptosis was easily distinguishable between septic and sham operated animals. While minimal apoptosis was detected early, pneumonia induced significant apoptosis 12 hours following the onset of infection, which stayed elevated at 24 hours (Fig. 3). Despite low levels of early apoptosis, higher levels of cell death were detected using H&E than the other three stains at 4 hours. H&E was also statistically higher than active caspase-3 at 12 hours.

#### *Anti CD3*

Mice had very low levels of gut epithelial apoptosis 4 or 12 hours following anti CD3 injection, but manifested a 10-fold increase 24 hours later (Figs. 4, 5). The delayed kinetics of gut apoptosis therefore contrasted greatly with either of the other injuries examined (compare Figs. 1, 3, 4). Despite low levels of apoptosis early, higher levels of cell death were detected using H&E compared to the other three stains at 4 hours, and H&E was statistically higher than active caspase-3 at 12 hours. When apoptosis increased markedly at 24 hours, TUNEL yielded a disproportionate increase in staining compared to the other methods utilized.

### **Discussion**

Since gut epithelial apoptosis has been demonstrated to play a critical role in the pathophysiology of multiple disease processes, there is substantial utility to reproducibly detecting intestinal cell death independent of injury, pathway of apoptosis, and timing of apoptosis appearance. While there is an extensive published literature looking at gut epithelial apoptosis using a variety of methods, a comprehensive comparison has not previously been done despite well documented limitations of each technique.

Of the nine combinations of injuries and time points examined in this study, there was only a single instance where the four stains quantitated were statistically similar to each other. There were six instances where a single stain was an outlier, defined as being statistically different from each of the three other stains (H&E three times, TUNEL twice, active caspase-3 once). There were also two comparisons where the only statistically significant difference was between H&E and active caspase-3 staining. At no time was ISOL an outlier stain.

A closer examination of the data demonstrates that

the TUNEL assay yielded substantially greater positive staining only in tissue where all methods of identifying apoptosis yielded markedly elevated levels of gut epithelial apoptosis (50 or greater positive cells/100 crypts). The fact that the TUNEL assay likely stains false positive cells was not surprising given the fact that this has long been described, whether in the intestine (Wolvekamp et al., 1998; Groos et al., 2003) or other epithelial tissue (Lesauskaite et al., 2004) despite the fact that it is used as commonly as any other stain for apoptosis. What appears to be unique here is the fact that the accuracy of the TUNEL assay seemed to track with the amount of apoptosis. When gut epithelial apoptosis was low, few cells were TUNEL positive, and this stain seemed as accurate as any other examined. However, when apoptosis was markedly elevated, the number of TUNEL positive cells far exceeded levels seen with the other stains (such as in early ionizing radiation or late anti CD3). If simply recognizing the presence or absence of apoptosis in the gut epithelium is the desired endpoint, the TUNEL assay may be an appropriate stain, but if accurate quantitation is needed, it appears to overestimate the true amount of apoptosis in severely injured intestine.

In contrast, active caspase-3 consistently demonstrated less staining than the other stains, regardless of injury or time point. This is consistent with data suggesting that compared to other techniques of apoptosis detection, active caspase-3 is unlikely to generate significant false positives, but is also likely to underestimate the actual amount of apoptosis. Depending on the sensitivity and specificity desired in quantifying gut epithelial apoptosis, active caspase-3 can therefore be considered an adjunctive stain but may underestimate the true amount of cell death.

The variability seen with H&E staining was somewhat surprising since H&E has previously been shown to have a reasonably good correlation with active caspase-3 staining in the gut epithelium in single injury studies (Marshman et al., 2001; Coopersmith et al., 2002b). In contrast, we found H&E to correlate to other techniques of detection far less commonly than any other stain used. We do not have a good explanation for this. Obviously, identifying apoptosis by morphologic criteria is subjective, but we minimized this by having the same examiner quantitate apoptosis on all sections in the study. Similarly, each data point presented represents an average of 20 sections, and the error bars for H&E are generally quite small, similar in size to those seen for the other stains. While our results do not indicate that H&E staining is inaccurate, they do suggest that H&E might not be appropriate to use as a sole method of quantifying gut epithelial apoptosis.

Of the stains studied, ISOL was never a statistical outlier. We cannot definitively state that this means it is more accurate than the other stains since there is no gold standard for quantifying gut epithelial apoptosis. However, regardless of injury, each stain demonstrated remarkably similar trends in identifying apoptosis (i.e. if cell death increased in one stain, it increased in all). This

## Apoptosis in the murine small intestine

greatly increases confidence in the accuracy of the data presented since these similarities were identified regardless of injury, regardless of pathway of apoptosis and regardless of time point studied. If the data presented accurately represent gut epithelial apoptosis at a given time point, they suggest that ISOL may be used as a single stain. Recent data suggests that ISOL is superior to TUNEL in identifying apoptosis (Lesauskaite et al., 2004), but to our knowledge this is the most comprehensive comparison of stains across a range of injuries, pathways of injuries and time points.

This study has a number of limitations. First we were unable to quantify apoptosis with either annexin V or M30. While there is limited data on the use of annexin V staining *in vivo*, we were unable to get staining above background, despite using published protocols (Bronckers et al., 2000; Paris et al., 2001; Bodiga et al., 2005) and greater than 15 additional modifications. We also saw scattered positive apoptotic cells with M30 staining, but this was markedly lower than that seen with the other stains used. Published data has shown that M30 staining can be accurate in rat intestinal tissue (Cinel et al., 2003; Bodiga et al., 2005), but this is not well described in murine gut. M30 staining has been reported to be sporadic in mouse tissue where elevated apoptosis can be documented by active caspase-3 (Perl et al., 2005), similar to the findings here. Another limitation to our findings is the assumption that being a statistical outlier means a stain is less accurate. It is based upon the assumption that a) if three stains yield similar results while a fourth yields different results that the fourth may be less accurate if used in isolation and b) if a stain is never a statistical outlier that it may represent the most accurate stain. However, in the absence of a gold standard to identify apoptosis, these conclusions cannot be definitively proved. Although we designed the study to look for results that were independent of both pathway of apoptosis and kinetics of apoptosis following injury, we also cannot definitively state that similar results would be found if examined in a different injury or at a different time point although the fact that our results were independent of these factors suggest they have wide generalizability. We also did not correlate our results to biochemical assays of gut epithelial apoptosis.

Despite these limitations, we believe this represents the most comprehensive study to date quantifying gut epithelial apoptosis. Based upon our results, we believe it is reasonable to use ISOL alone when quantifying intestinal cell death. Active caspase-3, H&E and the TUNEL assay are all useful when used in conjunction with another stain but should not be used to quantify gut epithelial apoptosis in isolation.

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