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Letter to the Editor

Detecting apoptosis during the formation of polarized intestinal epithelium in organ culture

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Dear Editor,

The intestinal epithelium is a constantly renewing tissue[1,2] that is maintained by coordinated patterns of stem cell renewal, cell proliferation, migration, differentiation and cell death. We have been studying a dynamic period during embryonic development (E13.5–E18.5) when the pseudostratified endoderm transforms into a differentiated, polarized monolayer of intestinal epithelium. Examination by light microscopy has revealed that superficial cells in the stratified epithelial cell layer degenerate and exfoliate during this period of development.[3,4] As the mouse embryo develops *in utero* and the intestine is an internal organ, this process is difficult to study *in vivo*. We have used an innovative embryonic gut culture system[5] in which the tubular architecture of the gut is maintained over several days and epithelial cells remain in contact with the underlying mesenchyme. This *in vitro* method has facilitated the investigation of the role played by apoptosis in the formation of intestinal epithelium.

Segments of mouse embryonic gut were dissected from the surrounding tissue at embryonic day 13.5 (E13.5) and attached to a filter paper before placement in catenary (suspension) culture in Terasaki wells (Figure 1a). At daily intervals, the explants were fixed and histology was used to examine the morphogenesis of the intestinal epithelium. In the early stages of culture, the endoderm cell layer was several cells thick and pseudostratified (Figure 1b). As the cultures progressed, it was evident that the epithelial cell layer was remodeling and cells exhibiting characteristics of apoptosis detached from the epithelium and were present in the lumen (Figure 1c, arrow). These cells exhibited all the hallmarks of cells undergoing apoptosis, including cell shrinkage and pyknotic nuclei. To observe apoptosis in this system, we used immunohistochemistry to detect the cleaved, activated form of caspase-3.[6,7] Multiple caspase-3 positive cells were identified in the lumen of the gut explants, confirming that the exfoliated cells were indeed undergoing apoptosis (Figure 1d, brown staining). The majority of cells within the epithelium were viable and negative for activated caspase-3. The pattern of cell death observed *in vitro* recapitulates that seen during normal intestinal development *in vivo* when the endoderm transforms into a monolayer of polarized epithelium.[3,4]

We also investigated the role of calcium-dependent cell adhesion molecules and medium containing serum on intestinal epithelial cell survival. Homophilic interactions between cadherin molecules require calcium ions.[8] To disrupt these interactions, explants of E14.5 midgut were perfused with EDTA and examined 24 h later for the presence of apoptotic cells (Figure 1e, f). Compared to controls (3.5% $\pm$ 3), there was a significantly higher percentage of apoptotic epithelial cells in explants treated with EDTA (19.5% $\pm$ 0.7, $P<0.05$). Many of the apoptotic cells detached and were present in the lumen. Epithelial cells in contact with the basement membrane survived the EDTA treatment (Figure 1e). This suggests that either the EDTA treatment did not fully penetrate the pseudostratified epithelial cell layer or that interactions with the extracellular matrix (ECM) supported survival. It is possible that cell survival is mediated by a mechanism similar to adult human intestinal epithelial cells, where integrins promote attachment to the ECM via focal adhesions and activate the prosurvival PI-3 kinase and Akt signal transduction pathways.[6,9,10]

Explants grown for 24 h in the medium containing minimal serum (0.5%) showed the most catastrophic level of cell death with large numbers of epithelial cells in the lumen staining positively for activated caspase-3 (Figure 1f). There were significantly more apoptotic epithelial cells (38.5% $\pm$ 7) compared to control or explants treated with EDTA ($P<0.005$). Many mesenchymal cells also died, indicating that all the cells in the explants required medium containing serum for survival.

The catenary method of gut culture contrasts with previous organ culture systems by maintaining the three-dimensional architecture of the gut such that the epithelial cell layer remains in contact with the basement membrane and surrounding tissue layers.[5,11] The technique allows the investigation of mechanisms underlying a normal developmental remodeling process in which some cells become susceptible to cell death and others survive. This differs from previous approaches where apoptosis is studied in detached intestinal epithelial cells.[6,8] We found that EDTA treatment or withdrawal of serum promoted epithelial cell death, which demonstrates that the level of apoptosis observed in the cultures can be manipulated by the addition or withdrawal of external agents. The role of specific pathways can be investigated by the addition of individual growth factors and inhibitors to the culture medium to modulate intestinal epithelial cell survival. The method could also incorporate analysis of tissues available from a wide variety of mutant mouse strains in which key components of apoptotic pathways, such as *Caspase* and *Bcl2* family members, are mutated.[12] This organ culture system is a significant technological advance that will contribute to the understanding of how the intestinal epithelium is first established during embryonic development and the roles played by specific

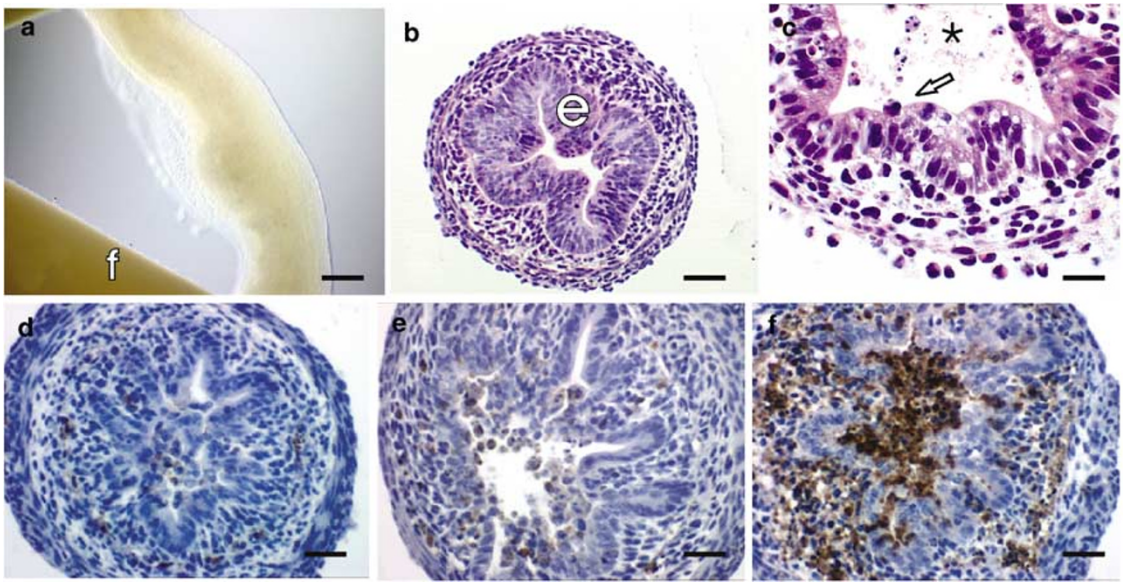

Figure 1 Monitoring apoptosis in explants of embryonic gut maintained in catenary culture. (**a**) Segment of embryonic gut attached to filter paper (f) and placed in catenary culture. The method was essentially as described previously.[5] E13.5 days postcoitus embryos were obtained from timed pregnancies of inbred ICR mice. Scale bar = 1 mm. (**b, c**) Transverse sections of embryonic gut cultured from E13.5 of development and stained with haematoxylin and eosin. (**b**) E13.5 midgut after 2 days in culture, Scale bar = 200 μm. (**c**) E13.5 midgut after 3 days in culture. Scale bar = 40 μm. (**d–f**) Embryonic E14.5 midgut fixed following 24 h in culture. Sectioned material was processed for immunohistochemistry for activated caspase-3 and stained with haematoxylin. Scale bars = 100 μm. (**d**) Control explant grown in the normal medium containing 15% fetal calf serum (FCS). (**e**) Explant grown in medium containing EDTA. For EDTA treatment, the lumens of segments of E14.5 gut were perfused with 5 mM EDTA prior to attachment to the filter paper. The explants were then cultured in normal medium for 24 h prior to fixation. (**f**) Explant grown in medium containing 0.5% FCS. In the serum withdrawal experiments, segments of embryonic gut were placed in medium containing 0.5% FCS for 24 h prior to fixation. For anti-active Caspase-3 immunohistochemistry, slides were dewaxed and rehydrated before incubation in Dako target retrieval solution (Cat No. S1700) for 20 min at 95–99°C. Slides were cooled for 20 min in the retrieval solution, washed several times in PBS and blocked in 5% FCS for 15 min. Sections were incubated in rabbit anti-human/mouse Caspase-3 Active IgG (R and D systems, 1/500) for 90 min at RT, then washed and incubated in 0.5% H_2O_2 in PBS for 15 min in order to block endogenous peroxidase activity. After further washing, the sections were incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Zymed) for 1 h. All slides were developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Dako) and lightly counterstained with haematoxylin prior to mounting in histomount (Zymed). Sections were viewed on a Zeiss Axioplan microscope and images were captured using a DAGE-MTI DC330 video camera and a Scion CG-7 frame grabber

signaling and apoptotic pathways in the formation and homeostasis of an epithelial monolayer.

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