The Involvement of Macrophages and Lymphocytes in the Apoptosis of Enterocytes

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Received March 27, 1995

Summary. Epithelial cells of the gut are characterized by rapid, constant cell renewal. The death of epithelial cells at the villus tips occurs so regularly that it must be regarded as a well-controlled cell death, designated as apoptosis. However, only limited information has been available on the mechanism of this phenomenon, including the disposal of the effete cells.

In the small intestine of the guinea pig and monkey, macrophages are densely aggregated at the lamina propria of the villus tips and vigorously engaged in the phagocytosis of effete epithelial cells. Intraepithelial lymphocytes possessing cytoplasmic granules, possibly intense in cytotoxicity, are topographically associated with the dying enterocytes, suggesting lymphocyte-mediated killing. After the engulfing of apoptotic enterocytes by macrophages, a thin apical portion of the enterocytes is left within the epithelium, maintaining the epithelial barrier until it is pinched off by the pushing of surrounding enterocytes.

In the rat and mouse, on the other hand, effete enterocytes are exfoliated as a whole from the villus tips into the gut lumen. Macrophages, also numerous at the villus tips in these species, are less intense in phagocytic activity. At the shoulder region of the villus, subepithelial macrophages extend thick processes deep into the epithelium; the processes appear to push out some enterocytes with typical apoptotic signs into the gut lumen. Lamina propria macrophages in the rat and mouse do not engulf enterocytes, but are believed to be involved in the induction of their apoptosis. The species difference in the mechanism of the apoptosis of enterocytes provides clues for understanding apoptosis.

Intestinal epithelial cells, originating in crypts, move toward the villus tip to be eventually extruded into the lumen (for review, see EASTWOOD, 1977; LEBLOND, 1981). Enterocytes (columnar cells or absorptive cells) are known to have a life span of 2 to 3 days in the mouse and rat, and 6–7 days in the human. The occurrence of apoptotic cell death in intestinal villi has not been dealt with in previous literature, except for GAVRIELI et al. (1992) who detected DNA fragmentation in situ in the epithelium of the villus tips. Apoptosis in the intestinal epithelium has been observed predominantly in the crypts (SEARLE et al., 1975) and in some special sites such as the lymphoid tissues of the appendix (SHIMIZU and WARREN, 1967). Our studies on the apoptosis of enterocytes started with our noting peculiar aggregations of lamina propria macrophages at the villus tips. This finding allowed an understanding of the complicated mechanism for the apoptosis of enterocytes. Moreover, we believe that a series of studies might give an answer to the question of whether or not the epithelial barrier of the intestine is destroyed when the effete epithelial cells are extruded.
phages in every villus suggests that they participate in important functions of the small intestine. Earlier anatomists considered that the macrophages at the villous apices might take up certain luminal substances passing through the epithelium (Von MÖLLENDORFF, 1925; MAXIMOW, 1927). On the other hand, SAWICKI et al. (1977), conducting autoradiographic and electron microscopic studies, have concluded that they might “play a role in the phagocytosis of some migrating cells of the intestinal mucosa, most probably the sheath-fibroblasts and/or intraepithelial lymphocytes.” The former cells, like enterocytes, have been shown to move from the bottom of villi towards the villus tips (PARKER et al., 1974).

Ultrastructurally, macrophages in the villus tips of the guinea pig small intestine are characterized by large phagosomes which constantly contain fragments of enterocytes undergoing different stages of digestion (HAN et al., 1993a), indicating that they continuously ingest cellular elements there (Fig. 2). Careful observation by electron microscopes has shown that the macrophages beneath the basal lamina extend their processes (pseudopodia) into the epithelium and engulf dead enterocytes, probably apoptotic bodies (HAN et al., 1993a). Moreover, a cell labeling method with bromo-deoxy-uridine (BrdU) revealed that labeled enterocytes moved from the crypts to the tips of villi with time, the labeled cells or their parts containing nuclei then appearing in the phagosomes of lamina propria macrophages (HAN et al., 1993a). This finding showed that effete epithelial cells are not exfoliated into the lumen, but phagocyted by the lamina propria macrophages. This hitherto unknown process sharply contrasts with the generally accepted view that aged enterocytes are simply shed into the lumen at the tips of villi (LEBLOND, 1981). Our novel concept is apparent also in the monkey and horse which display a similar aggregation of large macrophages at their villus tips (IWANAGA et al., 1992; HAN et al., 1993a) (Fig. 1). Noteworthily, such an image does not appear in the rat and mouse (HAN et al., 1993b), two of the most favored animals for studying the cell kinetics of enterocytes.

At the villus tip of the guinea pig small intestine, enterocytes are fragmented into several membrane-bounded bodies containing a nucleus and cell organelles. Their nuclei frequently show a condensation of the chromatin along the nuclear envelope (HAN et al., 1993a). These findings are images unique for programmed cell death, termed apoptosis (WYLLIE et al., 1980). Apoptotic cell death is known to be accompanied by DNA fragmentation into oligonucleosomes. When the TdT-mediated nick end-labeling technique (TUNEL) method (GAVRIELI et al., 1992) detecting fragmented DNA in situ is applied to the intestine of guinea pigs, labeled nuclei are numerous found in the cytoplasm of macrophages (Fig. 3). Labeled enterocytes are also found in the epithelium, although their incidence is considerably low. Therefore, this histochemical method should confirm our idea that lamina propria macrophages phagocytose the apoptotic enterocytes.

Apoptotic cells formed in the epithelia are generally believed to be either extruded into the lumen or phagocyted, mainly by adjacent epithelial cells and partly by macrophages (WYLLIE et al., 1980). Disposing of the apoptotic enterocytes observed in the guinea pig seems a unique process, because the cells are engulfed by macrophages located in the lamina propria.

REMOVAL OF APOPTOTIC ENTEROCYTES AND THE EPITHELIAL BARRIER

In the guinea pig, cell bodies of the effete enterocytes are phagocyted by lamina propria macrophages after apoptosis. However, the thin apical cytoplasm of enterocytes equipped with microvilli remains within the epithe-
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The skin-like apical cytoplasm, which is membrane-bound, is tightly connected to surrounding intact enterocytes via junctional complexes. The thin apical cytoplasm appears normal in structure, compared with that of adjacent enterocytes, except for the elongation and branching of the microvilli. It is not peculiar that cells can function in spite of the loss of the major part of cell bodies including the nuclei, as well-known examples of this are found in thrombocytes and red blood cells.

The elongated and branched microvilli are reminiscent of stereocilia, which are believed to be modified microvilli. Although the precise mechanism and significance of the morphological changes remains unknown, it may be caused by the disorder of cytoskeleton (IWANAGA et al., 1993).

The apoptotic enterocytes are easily identified by surface views using scanning electron microscopy (SEM) (Fig. 4). Cells covered with elongated and irregularly arranged microvilli are dispersed over a broad area of the villus tip (IWANAGA et al., 1993). This finding contrasts with the previously prevailing view that effete epithelial cells drop off in a group at one site of the summit of the intestinal villi (LEBLOND, 1981). At the following stages, the skin-like apical pieces are pushed by surrounding epithelial cells and extruded into the lumen, forming a dome-shaped or polypous protrusion. Their separation from the epithelium takes place after adjacent epithelial cells have closed defects in the epithelial lining by forming new junctional complexes beneath them (IWANAGA et al., 1993) (Fig. 5). Thus, the epithelial barrier in the villus tips is never destroyed during the renewal of epithelial cells.

Fig. 2. Electron micrograph showing a villus tip of the guinea pig ileum. Many macrophages (M) are densely packed in the subepithelial region. They are rich in phagosomes which frequently contain apoptotic nuclei (arrows). ×2,200
The mechanism for the disposal of apoptotic enterocytes can be easily and reliably confirmed by observations of luminal cell elements (HAN et al., 1993b). The precipitates of the fluid collected by rinsing the intestinal lumen in guinea pigs contain cytoplasmic fragments with the microvillous portion, but never contain a nucleus. The rich existence of such cytoplasmic fragments without the nuclei are also observed in the lumen of the monkey and horse small intestine.

LYMPHOCYTE-INDUCED APOPTOSIS OF ENTEROCYTES IN THE GUINEA PIG

Recent advances in the studies of apoptosis show that apoptosis is also inducible by various pathological conditions and endogenous and exogenous factors, which may include ischemia, radiation, toxins, and bioactive substances such as tumor necrosis factor (TNF). Even natural killer (NK) cells have been known to kill tumor cells in a mode of apoptosis (ZYCHLINSKY et al., 1991). It may by reasonable to consider that the apoptosis of enterocytes is well-regulated by certain external factors derived from lymphocytes which are rich in the villus tips.

Ultrastructural observations demonstrate numerous lymphocytes in the epithelium of villus tips (Fig. 6). The intraepithelial lymphocytes (IELs) are characterized by electron-dense granules in the cytoplasm, and are identifiable as large granular lymphocytes (LGLs) (IWANAGA et al., 1994b). LGLs correspond to a heterogeneous cell population including NK cells, cytotoxic T lymphocytes (CTLs) and lymphokine-activated killer (LAK) cells (KANEDA, 1989). Cytoplasmic granules of the lymphocytes contain cytotoxic substances including perforin (PODACK et al., 1985), a family of serine esterases (MUNGER et al., 1988; HAYES et al., 1989; ZUNINO et al., 1990), and TIA-1 (TIAN et al., 1991) as effector molecules which kill target cells. LGL-mediated lysis is accompanied by the fragmentation of the taget cell DNA, characteristic of apoptosis (PODACK and KONIGSBERG, 1984).

Perforin, one of the predominant cytotoxic substances contained in the granules of LGLs, is a bioactive peptide which forms in its target cell nonspecific ion channels through which markers of intracellular components can readily pass (HENKART et al., 1984; PODACK et al., 1985). The formation of these ion channels effectively induces the cell death of target cells. It is known that perforin induces cell lysis or membrane damage in target cells but not DNA fragmentation. On the other hand, granzymes and fragmentin have been shown to rapidly induce the fragmentation of DNA. Noteworthily, these two proteins are active only in the presence of perforin (HAYES et al., 1989; SHI et al., 1992). Moreover, the intensity of their cytotoxic activity is shown to be dependent on perforin-dosage, with DNA damage increasing with perforin concentration. The role of perforin in apoptosis is explained by the elevated permeability or stimulated endocytosis of serine esterases such as fragmentin into target cells (SHI et al., 1992).

Immunohistochemistry using anti-perforin antibody demonstrated perforin-containing lymphocytes in the epithelium of villi in the guinea pig (IWANAGA et al., 1994b). They are dispersed in the epithelial lining, with a tendency to be more numerous in the apical half of the villus. In contrast, the perforin-containing lymphocytes

Fig. 3. In situ nick end-labeling technique. Labeled cell elements in the ileum of a guinea pig are found exclusively in macrophages located in the lamina propria of villus tips (a). In the ileum of a rat (b), epithelial cells in both the villus tips and intestinal lumen are labeled. ×350
Fig. 4. Scanning electron microscopic view of a villus tip in the ileum of a guinea pig. Four apoptotic cells, identifiable as polypous protrusions, are scattered at the villus tip. ×3,000

Fig. 5. Electron micrograph showing the separation of a round apical cytoplasm, equipped with a microvillous portion, from the epithelium (guinea pig ileum). ×5,800
are very rare in the lamina propria. The IELs have been shown by immunologists to represent a special type of T cell bearing γδ receptors, which are able to detect and destroy damaged epithelial cells (JANEWAY et al., 1988; VINEY et al., 1990). On the other hand, αβ T lymphocytes are more numerous in the lamina propria and recirculate continuously from blood to lymphoid organs.

The cytotoxicity of LGLs in the intestinal epithelium is also suggested by their cytoplasmic processes deeply interdigitating with the enterocytes (IWANAGA et al., 1994b; TAKAHASHI-IWANAGA et al., 1995) (Fig. 6). The interdigitation of these lymphocytes with target cells has been spotlighted as an important sign of cytotoxic activity in cells (SANDERSON and GLAUERT, 1979). Their branched processes pierce into the enterocytes, which may show sign of apoptosis. The fragmentation or pinching-off of the enterocyte cytoplasm is frequently seen at the villus tips, presumably due to the invasion of LGLs into the epithelium. Characteristically, some lymphocytes which are buried in the enterocyte cell bodies separate the brush border region from the cell body, resulting in the formation of a skin-like apical cytoplasm. Recently microcinematographic observations have demonstrated the alternate protrusion and withdrawal of processes of lymphocytes against enterocytes (TAKAHASHI-IWANAGA et al., 1995). These findings suggest that the apoptosis of enterocytes may be induced by the intraepithelial cytolytic lymphocytes.

**APOPTOSIS OF ENTEROCYTES IN OTHER MAMMALS**

In the rat, observations of luminal cell elements demonstrate the predominant existence of cells possessing a rich cytoplasm and a nucleus in the luminal fluid (HAN et al., 1993b). The intraluminal cell elements are able to be identified as enterocytes from their closely arranged microvilli and ultrastructural characteristics of cell organelles. The nucleus of the cells displays both the compaction and segregation of the chromatin at the periphery, a figure characteristic of apoptosis, suggest-
ing the direct exfoliation of apoptotic enterocytes from the epithelial lining (cf. Fig. 3b). In the mouse, ultrastructures of the luminal cell elements are essentially identical to those in the rat. Therefore, the general view concerning the fate of effete epithelial cells (LEBLOND and MESSIER, 1958) holds true in these animals. The difference in the fashion of the disposal of apoptotic enterocytes between the guinea pig and the rat/mouse may be partially linked to the aggregation of macrophages at the villus tips. Macrophages in the rat, though numerous, do not show typical phagocytic signs, and those in the mouse are very few in number.

In the rat and mouse intestine, polypous protrusions of apoptotic enterocytes are frequently observed at the villus tips (IWANAGA et al., 1994a). Such protruded cells have lost contact with the basal lamina and possess a nucleus showing typical apoptotic changes. Another site of the exfoliation of apoptotic cells is seen at the “shoulder” of the villus, namely the base of the tapering end-portion of the villus.

Histochemistry for acid phosphatase demonstrates an abundance of macrophages in the lamina propria of villi in the rat small intestine. Macrophages are stellate cells dispersed in the lamina propria; they are more numerous at the upper half of villi. The macrophages do not show any phagocytic signs, based on the morphological findings of cathepsin B immunohistochemistry and electron microscopy (IWANAGA et al., 1994a). The inactive phagocytic activity of the macrophages may be reasonably considered, since they do not take up apoptotic enterocytes as do these cells in the guinea pig. One striking figure observed is the invasion of long columnar processes of macrophages into the epithelium. This figure is more frequent in the shoulder region of villi where the macrophages tend to gather subepithelially (Fig. 7). The topographical relation of macrophage processes and enterocytes is reminiscent of that of IELs and apoptotic enterocytes documented in the guinea pig.
The cytotoxicity of the lamina propria macrophages against enterocytes may be explained by a dual mechanism, namely mechanical and chemical attacks. First, invasion of the cytoplasmic processes (Fig. 7) may damage the enterocytes. Eroded areas of the epithelium are usually accompanied by the invasion of macrophage processes. We encountered apoptotic enterocytes which were underlain by the macrophage processes, looking as if the macrophage processes had pushed out the enterocytes. Second, macrophages produce and secrete several cytotoxic substances such as interleukins, superoxide radicals and nitric oxide. Tumor necrosis factor (TNF)-α, one of the predominant cytokines produced by macrophages, is known to induce apoptosis in target cells (SUDA et al., 1993; OHNO et al., 1993). Superoxide radicals and certain proteases have been found to bring about enterocyte damage in the animal model of Giardia lamblia (GOYAL et al., 1993). Identification of the cytotoxic substances produced by the lamina propria macrophages is an important subject for future studies.

The mechanism of the disposal of effete epithelial cells in humans remains unknown. Our experiences imply that the aggregation of macrophages rich in phagosomes is a reliable sign of the transportion of effete epithelial cells into the lamina propria. In the small intestine of humans, however, no marked aggregations of macrophages comparable to those in the guinea pig are recognizable, suggesting the shedding of enterocytes into the gut lumen. Recently, evidence for this idea has been offered by SHIBAHARA et al. (1995). However, the large intestine in humans displays aggregations of round, PAS-positive macrophages at the subepithelial region (YUNIS and SHERMAN, 1970). NAGASHIMA et al. (1993) reported that lamina propria macrophages in the human large intestine, especially in the colon, contained several epithelium-associated antigens such as cytokeratin and BerEP4; any macrophages in other tissues did not reveal the reactivity to these antigens. Therefore, the human intestine seems complicated with regard to the mechanism of the disposal of effete epithelial cells, because this appears rat-type in the small intestine, but guinea pig-type in the large intestine.

The species differences in the fate of aged enterocytes is worth noting, since the cell kinetics of intestinal epithelial cells is one of the most fundamental and important phenomena. The identification of factors regulating the apoptosis of enterocytes in various mammals is expected to serve our understanding of the mechanism of apoptosis, and also to effect a breakthrough in studies on the pathogenesis of intestinal diseases such as ulcerative colitis, Crohn's disease and melanosis coli.

REFERENCES


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