Lessons From Genetically Engineered Animal Models

VII. Apoptosis in intestinal epithelium: lessons from transgenic and knockout mice

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Apoptosis plays an important role in homeostasis of intestinal epithelia and is also a stress response to toxic stimuli. Transgenic and knockout mice have provided insights into the regulation of intestinal epithelial apoptosis that could not have been obtained by cell culture techniques. Two broad types of apoptosis have been characterized: spontaneous apoptosis, which occurs continuously at low levels in the normal, unstressed intestine, and stress-induced apoptosis, which occurs after genotoxic insult such as exposure to gamma radiation or DNA-damaging drugs. Spontaneous apoptosis occurs at the base of the crypt at or near the position of epithelial stem cells. Knockout studies have shown that spontaneous apoptosis is independent of p53 and Bax in both small and large intestine, whereas Bcl2 only regulates spontaneous apoptosis in the colon. Little is known about the regulation of the specialized form of cell death at the villus tip. In contrast, knockout studies have demonstrated that both p53 and Bcl2 are important regulators of stress-induced apoptosis, but that there are significant differences between early and late time points. Bax plays only a minor role in the regulation of stress-induced apoptosis. The cumulative effect of stress-induced apoptosis on tissue architecture is not straightforward, and cell cycle arrest also plays a critical role.

It has been known for several decades that programmed cell death is essential for the growth and development of multicellular organisms. However, it was not until 1972 that Kerr, Wyllie, and Currie (10) coined the term “apoptosis” and described its defining morphological features. Since then, it has become clear that apoptosis is also important in the development of the immune system, is a major mechanism of immune-mediated cytotoxicity, and participates in deletion of cells with potentially carcinogenic mutations. In disease pathogenesis, apoptosis can be either inappropriately excessive or deficient and has been implicated in a wide variety of gastrointestinal conditions including Helicobacter-associated gastritis, Shigella flexneri dysentery, inflammatory bowel disease, and colorectal neoplasia. Furthermore, anticancer drugs and nonsteroidal anti-inflammatory drugs induce apoptosis (25).

Apoptosis plays an important role in determining the architecture of intestinal epithelia and is also a part of the stress response of intestinal epithelial cells to toxic stimuli. Rather than being a single process, a number of mechanistically distinct pathways to apoptosis can be discerned in intestinal epithelia, which depend on the physical position of the cell along the crypt/villus axis, its level of differentiation, and the type of stimulus involved.

The study of apoptosis in gastrointestinal epithelium has been greatly hampered by slow progress in the development of suitable in vitro models of normal intestinal epithelial cells. All established gastrointestinal epithelial cell lines are abnormal by definition as they are immortalized with a disturbed balance between rates of cell death and proliferation. Such cell lines probably have abnormal apoptosis mechanisms. Also, many of the more commonly used cell lines are also fully transformed. Furthermore, the gastrointestinal epithelium is a complex tissue in which epithelial cells undergo a differentiation program and receive signals from extracellular matrix, neighboring cells, and circulating hormones, all of which potentially influence apoptosis. For these reasons, study of the regulation of apoptosis under normal physiological conditions has been largely restricted to techniques based on analysis of histological sections of intact...
epithelium. In this themes article, we shall review the
information gained on the regulation of apoptosis from
transgenic and knockout mice.

STRUCTURE AND CELL KINETICS OF INTESTINAL
EPITHELIUM

The small intestine provides a unique opportunity for
studying the influence of differentiation pathways on
apoptosis as cells at different stages of differentiation
can be identified simply through their position along
the crypt/villus axis. The intestinal epithelium is a
self-renewing monolayer arising from stem cells located
at or near the base of crypts (22). One can identify
their position by counting cell positions from the base of
the crypt. In the small intestine, stem cells are believed
to be located at cell positions 3–5, whereas in the large
intestine they are located at cell positions 1–2. Unfortu-
nately, there are no biochemical or molecular markers
for intestinal stem cells and their positions have been
implied from indirect experimental techniques and
mathematical modeling (22). Daughter cells undergo
division four to six rounds of cell division to form a cohort
of transit cells that populate the midportion of the crypt.
These cells differentiate into four cell lineages. Absorp-
tive enterocytes, comprising 80% of all epithelial cells,
goblet cells, and enteroendocrine cells all continue to
migrate up the crypt. Paneth cells are a fourth lineage,
located at the base of the crypt below the putative
position of stem cells. As cells exit the crypt onto the
villus, they stop cycling and become trapped in the G1
phase of the cell cycle as a result of downregulation of
cyclin D1 and cyclin-dependent kinase 2 (2). As they
migrate up the crypt, they continue to differentiate and
express a new repertoire of proteins such as brush-
border hydrolases. After 2–3 days, they reach this
villus tip where, in the mouse, they are shed at a rate of
1,400 cells · villus⁻¹ · 24 h⁻¹ (22). Thus the intestinal
epithelium has one of the most rapid turnover rates
among mammalian tissues.

GENETIC REGULATION OF APOPTOSIS IN INTESTINAL
EPITHELIUM

Mice that have been rendered homozygously null for
genes that regulate apoptosis have proved extremely
valuable in determining the mandatory role of a protein
in controlling apoptosis in intestinal epithelium. Experi-
ments involving these mice have often provided evidence
that confirms previous immunohistochemical studies,
yet these experiments have the additional advantages of
allowing damage-induced apoptosis to be easily studied
while avoiding some of the technical problems associated with the immunohistochemical
process.

Investigations of the levels and cell positions of
spontaneous apoptosis can provide evidence for the
roles played by these genes during homeostasis within
the epithelium. In addition, studies of the apoptosis
induced in the epithelium by gamma radiation and
cytotoxic drugs can demonstrate how these genes influ-
ence the response of the intestine to damage. Genotoxic
stimuli can also be used to investigate whether the
genes that regulate acute apoptosis also modulate the
long-term histopathological outcome of the epithelium
in response to cytotoxic damage. One note of caution
should be mentioned when interpreting any experi-
ments involving “knockout” mice, namely, that any
observed effects may have arisen not only from absence
of the gene being investigated but also from compen-
satory changes in other gene products, which may have
occurred during the animal’s development. For ex-
ample, Bcl2 knockout mice have severe developmental
abnormalities of the kidneys, causing renal failure (19).
It is at least theoretically possible that abnormalities in
apoptosis in these mice are due to the sequelae of renal
failure rather than to a direct result of Bcl2 deficiency.

To date, knockout mice have been used to determine
how p53 and two Bcl2 family members, Bcl2 and Bax,
affect both spontaneous and damage-induced apoptosis
in intestinal epithelia (see Table 1). Obviously, these
genes are likely to represent only the tip of the iceberg,
and similar experiments using a range of other knockout
mice will be necessary to establish a comprehensive picture
of gene-regulating apoptosis in intestinal epithelium.

APOPTOSIS MECHANISMS IN THE UNSTRESSED
INTESTINE

In the unstressed intestine two apoptotic pathways
have been identified. The first takes place in the crypt
at the level of the stem and early transit cells and is
sometimes referred to as “spontaneous” apoptosis, al-
though in fact the trigger has not been identified. This
spontaneous apoptosis takes place at a low rate and is
thought to regulate the number of cells entering the
crypt/villus axis. It is readily identified as apoptotic
bodies in histological sections fixed in Carnoy’s medium
stained with hematoxylin and eosin. Mice that are p53
deficient (7) show similar levels of spontaneous apopto-
sis in the intestinal epithelium compared with their
wild-type counterparts (3, 16). This suggests that p53
plays little part in normal homeostasis in this tissue.

Homozygous Bcl2-null mice (19, 20) demonstrate
levels of spontaneous apoptosis in small intestinal
crypts similar to their wild-type counterparts. In the
colon, however, Bcl2−/− mice show elevated levels of
spontaneous apoptosis, and this is concentrated at cell
positions 1–2 at the base of the crypt (17). This is the
location of Bcl2 protein expression found by immunohis-

Table 1. Genetic determinants of spontaneous
and stress-induced apoptosis in the
murine intestinal epithelium

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>p53</th>
<th>Bd2</th>
<th>Bax</th>
</tr>
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<tbody>
<tr>
<td>None (spontaneous)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Gamma radiation</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Ischemia-reperfusion</td>
<td>−</td>
<td>+</td>
<td>NK</td>
</tr>
</tbody>
</table>

NK = Not known.
tochemical studies and is also the presumed location of colonic stem cells. This observation suggests that Bcl2 expression plays a part in homeostasis of normal colonic epithelium, although its precise role remains to be established, since the actual morphology of the colonic epithelium is normal in Bcl2−/− mice (19, 20). In contrast, Bax expression appears to have little effect on homeostasis in normal intestinal epithelium, as Bax−/− mice (11) show the same levels of spontaneous apoptosis as their wild-type counterparts in both small intestine and midcolon (24). This correlates well with the observed immunohistochemical distribution of Bax expression in those small intestinal cell lineages that are not crucial for maintenance of epithelial renewal, namely, differentiated Paneth cells and villus enterocytes (13).

Apoptosis may also participate in the shedding process at the villus tip (for review, see Ref. 14). Careful studies with electron microscopy have demonstrated that neighboring epithelial cells extend processes underneath the cell to be extruded and form a tight junction. This tight junction then migrates toward the lumen like a “zipper,” pushing the cell to be shed out of the monolayer. Apoptotic morphology is only seen rarely during this extrusion process; the shed cells only take on apoptotic morphology once free in the lumen (27). It is not known at which point along the crypt/villus axis the process of apoptosis begins. This is because most of the important signal transduction events take place while the cell is morphologically normal. Nevertheless, at least some early apoptotic events must take place while the cell is within the monolayer, since activation of caspase-3, a member of the principal effector enzyme family of apoptosis, takes place at the villus tip (21).

Few data are available on the regulation of cell loss at the villus tip. It is clear that this shedding process is tightly regulated. It is unknown whether apoptosis itself is the regulated event or whether apoptosis is merely secondary to detachment from basement membrane. Bcl2 does not appear to regulate cell shedding, as villus dimensions are normal in mice with forced overexpression of Bcl2 in villus epithelial cells (6). A particularly fascinating point is that the rate of cell loss at the villus tip must be tightly coupled with cell production at the base of the crypt. This implies that there must be a feedback loop between the crypt and villus tip, but the anatomical basis for such a feedback loop is unknown. One possibility is that γ/β T cells migrating from the villus tip act as messengers to the crypt cells, as knockout studies have demonstrated that they are capable of reducing crypt cell production rate (12). Glucagon-like peptide-2 (GLP-2) is another candidate for regulating the shedding process on the villus. Raised levels of GLP-2 are known to increase villus length, possibly through inhibition of apoptosis at the villus tip and increased proliferation in the crypt (28). Very recently, the human and mouse GLP-2 receptor has been cloned and characterized as a member of the G protein-coupled receptor superfamily (18). It is unknown whether this receptor participates in the regulation of apoptosis.

Apoptosis is never seen along the length of the villus of the unstressed intestine except under highly artificial conditions. This is not because the villus cells are postmitotic, since forcing villus cells back into cycle through the overexpression of SV40 large T antigen does not restore radiation-induced apoptosis (5). Mice that express a dominant negative N-cadherin in their villus cells have spontaneous apoptosis along the length of the villus in addition to developing an inflammatory bowel disease reminiscent of Crohn’s disease and colonic adenomas (9).

STRESS-INDUCED APOPTOSIS IN INTESTINAL EPITHELIUM

p53. Studies of the apoptosis induced in p53−/− mice by administration of genotoxic stimuli have revealed that p53 is a crucial determinant of the apoptosis that occurs within a few hours of damage. The first observations of damage-induced apoptosis in this setting demonstrated that p53−/− mice showed absence of intestinal apoptosis, which is normally observed in p53 wild-type animals during the 3-4 h period following gamma radiation (3, 16). This observation correlated well with immunohistochemical studies in wild-type mice following the same stimulus of gamma radiation, since analysis of the cell positional distribution of immunohistochemical p53 protein expression was coincident with the position of apoptotic cells in the small intestine following 8-Gy gamma radiation (16).

The acute apoptosis induced within a few hours of gamma radiation was therefore demonstrated to be completely p53 dependent. What, however, was the long-term fate of the p53-null cells that had undergone significant DNA damage but had not been immediately deleted by apoptosis? Histological assessment of small intestinal epithelium 24 h after gamma radiation showed that significant numbers of apoptotic cells were present after 8 Gy, but very few were present after the lesser stimulus of 1 Gy. With the use of electron microscopy, the apoptotic cells of the p53-null mice seen 24 h after 8-Gy gamma radiation in p53−/− mice suggested that these cells might have arisen as a result of aberrant mitosis (4, 15). p53-independent mechanisms of apoptosis therefore exist to delete cells that have been damaged by high doses of radiation, but this p53-independent apoptosis is observed several hours later than the p53-dependent wave of apoptosis.

p53 expression also affects the acute intestinal apoptosis induced within 24 h by the alternative stimulus of the chemotherapeutic drug 5-fluorouracil, since p53-null mice show many fewer apoptotic cells than their wild-type counterparts after administration of this agent (26). However, the apoptosis induced by 40 mg/kg 5-fluorouracil was not influenced by coadministration of 500 mg/kg thymidine but was significantly rescued by 3,500 mg/kg uridine given 2 h after the cytotoxic stimulus. This suggests that the p53-dependent apoptosis induced by 5-fluorouracil occurs as a result of the RNA-damaging mechanism of the drug rather than as a result of DNA damage consequent on thymidylate synthase inhibition. This was confirmed by experi-
ments utilizing the pure thymidylate synthase inhibitor Raltitrexed (Tomudex, ZD-1694), which has no RNA-damaging effects. The apoptosis induced in small intestinal epithelium by this drug was completely rescued by coadministration of 500 mg/kg thymidine, yet was p53 independent, with no difference in apoptotic yield observed between p53 wild-type and null mice (Ref. 26 and Pritchard, unpublished observations). This finding also illustrates that the intestinal apoptosis induced by cytotoxic agents is not always p53 dependent and is influenced by the mechanism of damage caused by the drug.

The experiments described above have demonstrated that the acute apoptosis induced within a few hours of administration of gamma radiation and 5-fluorouracil is exquisitely p53 dependent. High doses of these cytotoxins also have long-term consequences in the intestine, manifested as histopathological changes such as villus shortening and ulceration and demonstrated clinically by reduced absorptive capacity of the gut and diarrhea. Does p53 expression also modulate this long-term response of the intestine to toxic damage? One way of investigating this has been to use clonogenic assay of gut integrity, as has been described above for the crypt microcolony assay. Such doses may completely overwhelm a cell’s metabolic responses, so that any differences that may have emerged with a less severe stimulus might remain hidden.

Bcl2 family members. The Bcl2 family consists of both pro- and anti-apoptotic proteins (1). A cell’s capacity to undergo apoptosis is probably determined by the ratio of all Bcl2 family members expressed within it. Knockout studies uniquely enable the contribution of an individual family member to be studied within the context of a complex tissue in vivo. Three to four hours following stimulation by gamma radiation, Bcl2−/− mice show similar levels of small intestinal apoptosis as their wild-type counterparts. However, as with spontaneous apoptosis, elevated levels of damage-induced apoptosis are observed in the colon. This colonic apoptosis again occurs specifically at those cell positions at the base of colonic crypts, which are believed to harbor the colonic stem cells (17). A similar picture is also seen using the alternative cytotoxic stimulus of 5-fluorouracil, in that Bcl2−/− mice demonstrate significantly greater levels of apoptosis particularly at the base of colonic crypts 4.5 h after 40 mg/kg 5-fluorouracil (24). However, 24 h after 40 mg/kg 5-fluorouracil administration, there was no significant difference in apoptotic yield between Bcl2 wild-type and null mice, suggesting that, in this setting, Bcl2 expression serves to delay the onset of apoptosis rather than prevent it completely (24). The long-term effects of the protection of stem cells at positions 1–2 at the early time points after 5-fluorouracil administration require a long-term toxicological study of gut integrity, as has been described above for p53−/− animals.

In contrast to p53 and Bcl2, Bax expression appears to play very little role in the regulation of damage-induced apoptosis in intestinal epithelium. Bax−/− mice show no significant difference in apoptotic yield compared with their wild-type counterparts in small intestine or midcolon 4.5 h after 1 Gy or 8 Gy of gamma radiation or 40 mg/kg 5-fluorouracil administration and only a small reduction in apoptotic yield 24 h after 40 mg/kg 5-fluorouracil administration (24). The contrast between the effects of Bax and p53 expression on the apoptosis induced in intestinal epithelium by both gamma radiation and 5-fluorouracil suggests that Bax expression is not an important determinant of the p53-mediated induction of apoptosis in this setting. Other downstream factors are thus likely to be responsible for the pro-apoptotic effects of p53 in this tissue following cytotoxic stimuli. Experiments similar to those described above have not yet been conducted in mice that have been rendered homozygously null for other members of the Bcl2 family. Hence, the effects of these gene products on spontaneous and damage-induced apoptosis in intestinal epithelium remain to be established.

Ischemia-reperfusion-induced apoptosis is another example of stress-induced apoptosis, which contrasts with radiation-induced apoptosis in revealing ways (6). The ischemia-reperfusion injury is caused by temporary occlusion of the blood supply followed by its restoration. This stimulus induces apoptosis in both
the crypt and villus and is independent of p53 but inhibited by Bd2. This tells us that epithelial cells on the villus have the molecular machinery to undergo apoptosis but for some reason it is not activated by radiation. One can speculate that apoptosis on the villus is induced by reintroduction of a blood borne factor, which is destroyed by radiation.

In conclusion, knockout studies have provided results that could not have been obtained from experiments utilizing either the cell lines available today or human samples. In addition, they have yielded mechanistic information that could not be gained from immunohistochemical studies. Nevertheless, they have significant disadvantages in that generating a single knockout strain is both time consuming and expensive. Numerous strains are necessary to dissect the complex interrelationships among all the apoptosis regulators relevant to intestinal epithelium. However, such studies are vital for the rational design of new and more effective therapies for major intestinal epithelial diseases, such as inflammatory bowel disease and cancer.

A. J. M. Watson is supported by grants from the Cancer Research Campaign, the Association of International Cancer Research, and North West Cancer Research Fund.

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