Apoptosis, inflammatory bowel disease and carcinogenesis: Overview of international and Greek experiences

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Apoptosis is critical for organ development, tissue homeostasis, the elimination of abnormal cells and the maintenance of immune homeostasis by variable regulatory mechanisms. The death of T lymphocytes following their activation involves a series of proteases (caspases), which comprise the central executioners of apoptosis. Abnormal regulation of apoptosis results in disease. T-cell resistance against apoptosis contributes to inappropriate T-cell accumulation and the perpetuation of the chronic inflammatory process in inflammatory bowel disease with potential tumourigenic effect. The use of antitumour necrosis factor-alpha, anti-interleukin-6R and anti-interleukin-12 antibodies suppresses colitis activity by induction of T-cell apoptosis, thereby having important implications for the design of effective therapeutic strategies in inflammatory bowel diseases. Contrary to international data, the incidence of cancer in Greek patients with inflammatory bowel disease appears to be low. A balance between cell proliferation (Ki-67 overexpression) and apoptosis (Bax protein overexpression) may partly explain the low incidence of cancer development in Greek inflammatory bowel disease patients.

Key Words: *Apoptosis; Carcinogenesis; Caspases; Inflammatory bowel disease*

Apoptose, maladie intestinale inflammatoire et carcinogenèse : Vue d'ensemble des expériences internationales et grecques

L'apoptose est essentielle au développement des organes, à l'homéostase tissulaire, à l'élimination des cellules anormales et au maintien de l'homéostase immunitaire suivant divers mécanismes régulateurs. La mort des lymphocytes T après leur activation met en jeu une série de protéases (caspases) qui constituent les principaux effecteurs de l'apoptose. Une régulation apoptotique anormale entraîne la maladie. La résistance des cellules T à l'apoptose contribue à une accumulation inappropriée de lymphocytes T et à la perpétuation du processus inflammatoire chronique de la maladie intestinale inflammatoire, avec un effet cancérigène potentiel. Le recours au facteur de nécrose tumorale alpha, à l'anti-interleukine-6R et aux anticorps anti-interleukine-12 supprime la colite par l'induction de l'apoptose des lymphocytes T, d'où ses répercussions importantes dans la l'élaboration de stratégies thérapeutiques efficaces pour la prise en charge des maladies intestinales inflammatoires. Contrairement à ce qu'on observe à l'échelle internationale, la fréquence de cancer chez les patients de nationalité grecque atteints de maladie intestinale inflammatoire semble faible. Un équilibre entre la prolifération cellulaire (surexpression du Ki-67) et l'apoptose (surexpression de la protéine Bax) pourrait expliquer en partie cette faible incidence de cancer chez les patients grecs souffrant de maladie intestinale inflammatoire.

For each cell there is a time to live and a time to die. There are two ways in which cells die. They are either killed by harmful agents (exposure to toxic chemicals and mechanical injury) or they are induced to commit suicide. During the process of death by injury (necrosis), the cellular metabolism breaks down, the cell swells, the cellular membranes decompose and the cell contents leak out, resulting in the inflammation of surrounding tissues. On the other hand, during the process of death by suicide (programmed cell death), the cell shrinks, its mitochondria break down with the release of cytochrome c (Cyt c), it develops bubble-like blebs on its surface, the chromatin (DNA and protein) in its nucleus degrades and breaks into small, membrane-wrapped, fragments (apoptotic bodies). Consequently, the phospholipid phosphatidylserine, which is normally hidden within the plasma membrane, is exposed on the cell surface, and is then bound by receptors on phagocytic cells (ie, macrophages and dendritic cells) which then engulf the cell fragments. Lastly, the phagocytic cells secrete cytokines that inhibit inflammation. It should be emphasized that, despite the widespread occurrence of apoptosis in physiological and pathological cell death, the occurrence of cell death that fulfils neither the criteria for apoptosis nor necrosis has been well documented (paraptosis, from para meaning next to or related to, and apoptosis) (Table 1) (1). Examples that do not conform to the criteria for either of these forms of cell death include a number of neurodegenerative cell deaths, such as those in a transgenic mouse model of amyotrophic lateral sclerosis, some developmental cell deaths, such as autophagic cell death and cytoplasmic cell death, or a number of ischemia-related cell deaths featuring cell swelling, referred to as oncosis. Such an alternative nonapoptotic form of cell death is programmatic, because it requires gene expres-

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TABLE 1
Comparison of apoptosis, necrosis and paraptosis

	Apoptosis	Necrosis	Paraptosis
Morphology			
Nuclear fragmentation	+	–	–
Chromatin condensation	+	–	±
Apoptotic bodies	+	–	–
Cytoplasmic vacuolation	–	+	+
Mitochondrial swelling	Sometimes	+	Late
Genomic effect			
TUNEL	+	Frequently –	–
Internucleosomal DNA fragmentation	+	–	–
Caspase activity			
DEVD-cleaving activity	+	–	–
Caspase 3 processing	+	–	–
PARP cleavage	+ (85 kDa fragment)	+ (50 to 62 kDa fragments)	–
Inhibition by:			
zVAD.fmk	+	–	–
BAF	+	–	–
p35	+	–	–
xiap	+	–	–
Bcl-x_L	+	Frequently –	–
Actinomycin D	Sometimes	–	+
Cycloheximide	Sometimes	–	+

DEVD Aspartic acid-glutamic acid-valine-aspartic acid; PARP poly(ADP-ribose) polymerase; TUNEL Terminal deoxynucleotidyltransferase-mediated uridine triphosphate end-labelling; + Present; – Absent

sion. Understanding the biochemical pathway(s) for nonapoptotic programmed cell death should lead to new insight into cell death events and their implications for the understanding of neurodegeneration, tumour therapeutics, development and evolutionary aspects of cell death programs (1).

There are two different reasons by which a cell commits suicide. First, programmed cell death is as important as mitosis in the development and maintenance of host homeostasis. For example, during embryogenesis, excess numbers of developing cells die and in hormone-responsive tissues, such as the uterus, cyclical depletion of specific hormones leads to the death of hormone-dependent cells (2). Similarly, the formation of the proper connections (synapses) between neurons in the brain requires that excess cells be eliminated by apoptosis (2). This concept implies that many cell populations have a propensity to undergo apoptosis (ie, are programmed to die unless specifically protected). Namely, cells must constitutively express, or try to express, molecules that function as effectors of apoptosis, and, moreover, these effectors are actively suppressed to allow the cells to survive (2). The second reason for which a cell commits suicide is that programmed cell death is required to destroy cells that represent a threat to the integrity of the organism. Examples include cells infected with viruses (in this situation cytotoxic T lymphocytes [CTLs] execute virus-infected cells by inducing apoptosis); and cells of the immune system. As cell-mediated immune responses decline, the effector cells must be removed to prevent them from attacking body constituents. Therefore, CTLs induce apoptosis in each other and even in themselves. Defects in the apoptotic mechanisms are associated with autoimmune diseases such as rheumatoid arthritis, lupus erythematosus or inflammatory bowel disease (IBD). Moreover, prolonged immune responses of the effector cells that exhibit defective apoptosis have been associated with chronic epithelial damage that can induce tumourigenesis in many tissues. In the gastrointestinal tract, inflammation has been linked to cancers in the esophagus (reflux disease and Barrett's esophagus), stomach, small bowel (Crohn's disease and adenocarcinoma) and colon (IBD and adenocarcinoma) (3).

Apoptotic procedure is implicated in tumourigenesis. Thus, some cancer-causing viruses use 'tricks' to prevent apoptosis of the cells they have transformed. For instance, two human papilloma viruses have been implicated in inducing cervical cancer, and one of them produces a protein (E6) that binds and inactivates the apoptosis promoter p53. Even tumour cells produced without the contribution of viruses may have 'tricks' to avoid apoptosis. For instance, some cancer cells, particularly lung and colon cancer cells, secrete elevated levels of a soluble 'decoy' molecule that binds to Fas ligand (FasL), plugging it so that it cannot bind Fas (CD95/APO-1). Therefore, CTLs cannot kill the cancer cells by the Fas-FasL apoptotic pathway. Lastly, other cancer cells express high levels of FasL, and can kill any Fas expressing CTL that tries to kill them.

In general, the process of CTL-mediated apoptosis (lysis) of target cells progresses in five key steps:

Step 1: *Target cell antigen recognition by CTL and conjugate formation between CTL and target cell*: The CTL binds to the target cell, by using its specific antigen receptor and other accessory molecules such as CD8, CD2, and leukocyte function-associated antigen-1 (LFA-1). Therefore, the recognition of the target cell involves class I major histocompatibility complex molecules (the ligand for CD8) complexed to a foreign peptide antigen on the surface of the target (eg, virus infected) cell, the complex serving as the ligand for the T-cell receptor (TCR), leukocyte function-associated antigen-3 (the ligand for CD2), and intercellular adhesion molecule-1 or intercellular adhesion molecule-2 (the ligands for LFA-1). Of note, transient conjugate formation may occur via CD2- or LFA-1-mediated adhesion in the absence of or before specific antigen recognition or antigen recognition may increase the ability to form conjugates by enhancing the binding function of the adhesion molecules (4).

Step 2: *CTL activation by clustering of its antigen receptor, initiated by recognition of major histocompatibility molecule-peptide complexes on the target cell*. The intracellular signals generated by the TCR may be increased by signals delivered through the various accessory molecules.

Step 3: *Delivery of lethal hit mediated by perforin and granzyme B and/or Fas-FasL complex*. During this process, CTLs develop specific membrane-bound cytoplasmic granules that contain numerous macromolecules, including a membrane pore-forming protein called perforin or cytolysin; enzymes frequently called granzymes that contain reactive serines in their active sites; and proteoglycans (4). In addition, CTLs share with other activated T lymphocytes (TLs) surface expression of FasL, which can deliver apoptosis-inducing signals to target cells expressing the Fas protein. Moreover, CTLs share with other effector TLs the ability to transcribe and secrete cytokines and other proteins upon activation, mainly tumour necrosis factor (TNF), interferon-γ (IFN-γ), lymphotoxin, and, to a lesser degree, interleukin (IL)-2. Studies of CTL-mediated killing of target cells in vitro had shown that granule exocytosis-dependent (ie, perforin/granzyme-mediated) killing and granule exo-

cytosis-independent (ie, FasL-mediated) killing were redundant. More recent reports have suggested that perforin and granzyme are the key mediators of CTL function in immune responses to intracellular microbes. In contrast, the Fas pathway appears to be more important for immune regulation than for CTL functions (ie, for controlling excessive lymphocyte activation, particularly against self-antigens).

The lethal hit is initiated by exocytosis of the granule contents onto the membrane of the target cell. As a consequence of granule content exocytosis, perforin, present as a monomer in the granule, comes in contact with extracellular levels of calcium (typically 1 to 2 mM) and undergoes polymerization. Polymerization of perforin leads to the formation of a large water-filled channel that preferentially occurs in a lipid bilayer, such as the plasma membrane of the target cell. If an adequate number of polymerized channels are assembled, the target cell will be unable to exclude ions and water, resulting in osmotic swelling and lysis. A second, equally important, function of perforin is to provide a means for introducing granzymes into the target cell. Granzyme B is a serine protease that preferentially cleaves protein substrates at aspartic acid residues. Target proteins that are recognized by granzyme B include IL-1 converting enzymes (ICE) or related cysteine proteases (caspases) that are themselves aspartic acid-directed. ICE and ICE-like proteases become catalytically activated upon cleavage at susceptible aspartic acid residues, so that by cleaving ICE, granzyme B initiates an ICE protease cascade that is comparable to the activation of the cascade induced by ligation of cell surface Fas. The net result is that the insertion of granzyme B into the cytoplasm of a target cell, like the ligation of Fas, initiates apoptotic cell death. Thus, by activating an apoptotic death pathway, a perforin/granzyme B-induced cascade of caspases can lead to the destruction of viral DNA genomes as well as the target cell genome, thereby eliminating potentially infectious DNA.

Step 4: *Detachment of CTL*

Step 5: *Death of the target cell by osmotic lysis and apoptosis (4).*

With regard to lymphocytes, programmed cell death plays a key role in controlling the size of the lymphocyte pool at several stages of lymphocyte maturation and activation. Immature lymphocytes that do not express functional antigen receptors undergo programmed death. After their maturation, if lymphocytes never encounter antigens, they die by apoptosis. Even if lymphocytes are activated by antigen, fractions of the progeny that do not receive sufficient growth factors or sustained stimulation also die. During lymphocyte maturation and activation, fluctuations in the levels of expression of *Bcl-2* or *Bcl-*x_L appear to correlate inversely with susceptibility to apoptosis (2). Overexpression of *Bcl-2* or *Bcl-*x_L leads to enhanced survival of immature lymphocytes and prolonged antibody responses. Conversely, knockout of *Bcl-2* or *Bcl-*x_L results in reduced survival of mature or immature lymphocytes. It has also been suggested that the long life span of memory lymphocytes may be due to constitutive expression of *Bcl-2* and/or *Bcl-*x_L (2). Besides, lymphocytes undergo a second form of apoptotic death, that occurs not because of lacking stimulation or deficient of growth factors but as a consequence of receptor-mediated activation. This is termed activation-induced cell death (AICD) and is considered to be induced and regulated by mechanisms that are different from those that control Bcl-2-regulated programmed cell death. In particular, AICD can be induced through activation of surface death receptors (such as Fas and TNF-receptor [TNFR]) (5,6). AICD is essential for the death of lymphocytes that recognize self-antigens and also plays a role in the induction of tolerance to some foreign antigens (2).

At the intestinal mucosal level, the apoptosis of lamina propria (LP) TLs is regulated via active and passive mechanisms. Immune responses in the mucosa are commonly characterized by major expansions of antigen-specific TLs that have potent effector function (7). Although this may be essential for host defense, it may also result in effector cell populations with considerable autoreactivity that can cause mucosal inflammation. To deal with this latter possibility, the mucosal immune system has evolved several strategies for the control of mucosal immune responses. Among these is the regulation of apoptosis that either occurs by an active mechanism following TCR stimulation (AICD) or by a passive mechanism following lymphokine (for instance, IL-2) withdrawal (7). The active mechanism involves death receptors such as Fas and TNFR and/or their ligands (FasL and TNF), while the passive mechanisms do not (7).

At the molecular level, disturbances in the regulation of these forms of apoptosis within the mucosal immune system, lead to mucosal inflammation that predispose to cancer development.

Death of intestinal TLs mediated by Fas-FasL

Activated T-cells are more susceptible to apoptosis than resting T-cells. As most T-cells in the LP normally exhibit a higher state of activation, and express memory cell markers such as CD45RO, the activation-induced cell death might be very important to downregulate effector cell function and cytokine production in the gut, thereby maintaining the immune homeostasis in the specialized microenvironment of the mucosa (7). Memory T-cells are known to express Fas constitutively at high levels, and normal LP T-cells (LPTs) exhibit an enhanced susceptibility to Fas-mediated cell death (7). Death-receptor-induced apoptosis comprises signaling processes via Fas, TNFRI, TNF-related apoptosis-inducing ligand-R1 and 2, and DR3 or DR6. Upon triggering of Fas by FasL, the adaptor molecule Fas-associated death-domain-containing protein (FADD) and pro-caspase 8 are recruited to the Fas receptor, thereby forming a death-induced signaling complex (DISC) (7). In particular, trimerization, or more likely oligomerization of Fas results to the recruitment of the Fas-adaptor protein, FADD, through their mutual death domains (DDs) (8). The other end of FADD contains two death effector domains (DEDs) through which it can recruit caspase 8 or its enzymatically inactive homolog and Fas inhibitor, FADD-like IL-1β-converting enzyme (FLICE) inhibitory proteins (8). Of note, Ced-3 gene encodes a protein related to mammalian, IL-1β-converting enzyme (ICE; caspase 1), which exerts proteolytic activation of the caspase cascade, thereby emerging as the most central step of apoptosis (9). To date, 14 mammalian caspases have been identified and involved in different aspects of cell death, although the exact function of each individual caspase is still mostly unidentified (9). Caspases are created as proenzymes containing an amino-terminal prodomain and the p20 and p10 domains that are cleaved to form the active enzyme as a tetramer of the two p20-p10 heterodimers; each tetramer contains two active sites (8). Caspases cleave precursors to create mature cytokines (caspase 1 [ICE, IL-1β converting enzyme] and caspase 11), thereby activating upstream

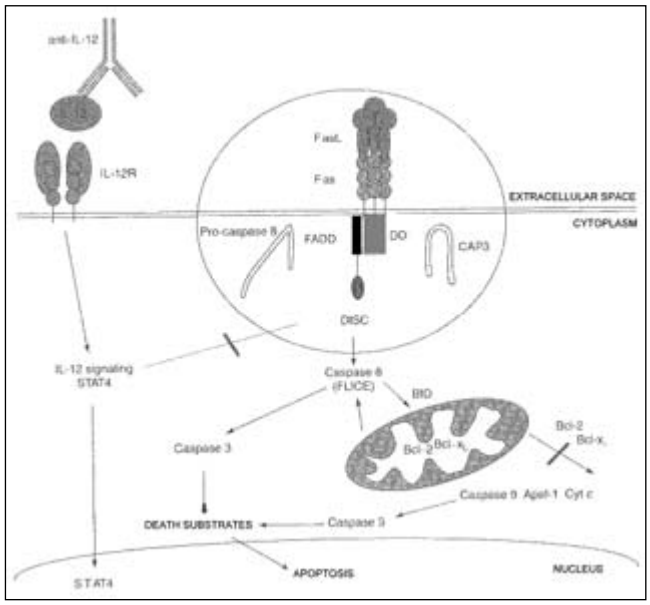

Figure 1) *Apoptosis of mucosal T-cell by the Fas-Fas ligand (FasL) system and suppression of Fas-mediated apoptosis by interleukin (IL)-12. Normal lamina propria T (LPT) cells demonstrate an enhanced susceptibility to Fas-mediated cell death. Upon activation of Fas, the proteins Fas-associated death domain (FADD), cytotoxicity-dependent Apo-1-associated protein 3 (CAP3) and pro-caspase 8 are recruited to the death domain (DD) of the receptor, forming the death induced signaling complex (DISC). DISC signaling events result in the activation of caspase 8 which, in turn, either directly activates caspase 3 or induces cytochrome c (Cyt c) release from mitochondria. Cyt c in the cytoplasm binds to apoptotic protease activation factor (Apaf)-1, forming a complex that binds caspase 9 and causes its activation. Subsequently, activated caspase 9 activates other proteases, including caspase 3, and therefore causes cleavage of various substrates that subsequently lead to the death of the cell. However, in chronic intestinal inflammation, increased production of IL-12 prevents Fas-mediated T-cell apoptosis, resulting to T-cell accumulation in the intestine. This mechanism of disease perpetuation can be successfully blocked by antibodies to IL-12 (7,13). STAT4 Signal transducer and activator of transcription 4*

(initiator) and downstream (effector) caspases. Initiator caspases, such as caspases 8 and 9, exert regulatory roles. Upon binding to signal-transducing molecules, they subsequently activate downstream effector caspases, such as caspases 3, 6, or 7, which finally cleave different cellular substrates, thereby inducing the apoptotic cell death (9-11). Moreover, there is recent evidence indicating a participation of caspases and other apoptosis-regulatory molecules beyond that of cell death, including the control of T-cell proliferation and cell cycle progression (9).

DISC signalling events result from activation of caspase 8 (recruitment of pro-caspase 8 leads to its autoproteolytic activation and the release of active caspase 8 [FLICE] into the cytosol). Of note, cytotoxicity-dependent Apo-1-associated protein 3 is identified as the FLICE prodomain which likely remains bound to the receptor after Fas-inducing proteolytic activity (12). Active caspase 8, either directly activates caspase 3 or induces Cyt c release from mitochondria. In the cytoplasm, Cyt c binds to apoptotic protease activating factor (Apaf)-1, forming a complex that binds caspase 9 and induces its activation. Consecutively, activated caspase 9 activates other proteases, including caspase 3, and thus induces cleavage of various substrates that subsequently leads to cell death and phagocytosis (7). Notably, active caspase 8, depending on its amounts, can trigger one of two different signalling pathways. The first pathway of Fas-mediated apoptosis is activated by low amounts of caspase 8 and involves the cleavage of the proapoptotic Bid molecule, followed by the release of Cyt c from mitochondria into the cytoplasm, and subsequent death of the cell (Figure 1) (7,13). The second pathway involves large amounts of caspase 8 that bypass mitochondria and activate other caspases such as caspase 3. The downstream smaller 'effector' caspases (caspases 3, 6 and 7) that are cleaved by active caspase 8, lack amino-terminal homoaffinity domains such as DDs, DEDs, or caspase recruitment domains but degrade a variety of cellular components including cytoskeletal proteins fodrin and gelsolin, nuclear lamins, and the inhibitor of caspase-activated DNase, which then permits activation of caspase-activated DNase to degrade DNA. Caspase 8 can also cleave the Bcl-2 homologue, Bid, to produce an active truncated Bid fragment that complexes with and inhibits Bcl-2 in the outer mitochondrial membrane to initiate the mitochondrial death sequence (8). Under normal conditions, Bcl-2 and Bcl-x_L insert in the outer mitochondrial membrane and appear to help maintain mitochondrial integrity by allowing the export of hydrogen ions from the intermitochondrial space, or perhaps through their capacity to form an ion channel. In contrast, other Bcl-2 family proteins (Bid, Bad, Bak, Bax, Bim, and Noxa) complex with Bcl-2 or Bcl-xL and inhibit their action (8). Disruption of the outer mitochondrial membrane allows the leakage of two critical mediators of apoptosis. The first is Cyt c, which can complex with Apaf-1 and recruit caspase 9, forming the apoptosome. In the presence of dATP this results in a conformational change in caspase 9 to render it active. The second mitochondrial factor is second mitochondria-derived activator of caspases, or direct inhibitors-of-apoptosis proteins (IAPs)-binding protein with low pI, which inactivates the IAP. This also results in activation of downstream effector caspases (8).

Of note, three groups have been identified in Bcl-2 family members. Group A contains antiapoptotic proteins such as Bcl-2 and Bcl-x_L that are characterized by four short conserved Bcl-2-homology (BH) domains (BH1-BH4), as well as a carboxy-terminal hydrophobic transmembrane tail domain, which localizes the proteins to the outer mitochondrial membrane and endoplasmic reticulum (ER). Group B members exhibit proapoptotic activity. This group includes Bax and Bak and resembles Group A except for the lack of the amino-terminal BH4 domain. Group C contains a large group of proapoptotic molecules (eg, Bid and Bik) that are much less similar than Groups A and B and contain only the BH3 domain (8).

The proto-oncogene *Bcl-2* is expressed in the mitochondrial membrane as well as in the contiguous membrane of the ER and nuclear envelope, mediating resistance against a wide range of apoptotic stimuli (11,14,15). The ER plays an important role in intracellular calcium homeostasis, and alterations in this homeostasis are implicated in the control of apoptosis. Bcl-2 decreases the free calcium concentration within the ER and suppresses apoptotic cell death (16,17). Moreover, experimental evidence suggests that Bcl-2 targeted to the ER can inhibit Myc-, but not etoposide-induced apoptosis in the rat-1 fibroblast cell line (14) On the other hand, the Bcl-2 family

members Bax and Bak involve direct effects on the ER calcium pool with consequent sensitization of mitochondria to calcium-mediated fluxes and Cyt c release. These effects modulate the kinetics of Cyt c release and promote apoptosis (18).

Because Bcl-2 and Bcl-x_L only inhibit apoptotic activities of mitochondria, the first (activated by low amounts of caspase 8) but not the second (activated by large amounts of caspase 8) pathway of active apoptosis is inhibited by these antiapoptotic proteins. On the other hand, passive forms of apoptosis involve the activation of Bid proteins that act exclusively via mitochondrial mechanisms; therefore, passive apoptosis, as caused by lymphokine withdrawal, is strongly inhibited by Bcl-2 and Bcl-x_L (7).

With regard to chronic intestinal inflammation, recent data indicate that increased production of cytokines (IL-12) prevent Fas-mediated T-cell apoptosis, resulting in T-cell accumulation in the gut. This mechanism of disease perpetuation can be successfully blocked by antibodies to IL-12 (7).

In summary, the death-receptor pathway as typified by Fas (CD95/APO-1) includes the following. Oligomerization of Fas by FasL (on the same cell or a neighbouring one) causes recruitment of FADD to the cytoplasmic tail of Fas by their mutual DDs. The opposite end of FADD contains DEDs that allow recruitment of either procaspase 8 or FLICE inhibitory proteins. The latter contains a mutation in the enzymatic domain rendering it enzymatically inactive. Caspase 8 can cleave the BH3-only protein, Bid, and the resulting truncated Bid can inactivate Bcl-2 in the mitochondrial membrane (as can Bax following DNA damage). This allows the escape of Cyt c, which clusters with Apaf-1 and caspase 9 in the presence of dATP to activate the caspase 9. Second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI is also released from the mitochondria and inactivates IAPs. Active caspase 9 can cleave and activate procaspase 3 to its active form, resulting in activation of other caspases, breakdown of several cytoskeletal proteins and degradation of the caspase-activated DNase inhibitor, thereby releasing caspase-activated DNase that leads to DNA degradation, cell death and phagocytosis of cell debris (Figure 1) (8).

Apoptosis of LP CD4+ T-cells in the normal and inflamed intestine

Naive T-cells are mostly Fas-negative and thus resistant to Fas-mediated apoptosis, unlike memory T-cells that are largely Fas-positive. As the majority of T-cells in the LP express memory cell markers, and, moreover, memory T-cells express high levels of Fas, in the unstimulated state (before TCR stimulation), LPT cells display increased susceptibility to Fas-mediated apoptosis compared with unstimulated peripheral-blood T-cells; this is due to as yet unknown downstream changes in the Fas signaling pathway. In addition, LPT cells show augmented spontaneous apoptosis compared with peripheral blood cells; this is possibly due to a passive apoptotic mechanism associated with IL-2 withdrawal, as apoptosis is decreased by addition of IL-2 and is induced by addition of anti-IL-2 antibodies. Similarly, after stimulation of LPT cells with anti-CD2 and anti-CD28 antibodies, apoptosis is further increased compared with stimulated peripheral blood T-cells (19). This augmented apoptosis is the consequence of activation-induced apoptosis involving death receptors, because it is observed when the cells are activated by the CD2 rather than the CD3 activation pathway, thereby CD2 pathway is a more effective means of activating T-cells (20). Besides, this apoptosis is extinguished by antibodies that block Fas, while addition of IL-2 or anti-IL-2 antibodies has a minor effect (7). In contrast to LPT cells from control patients, those from patients with ulcerative colitis and Crohn's display defective Fas-induced apoptosis upon stimulation by CD2. Even though LPT cells in IBD express the similar amount of Fas on their surface, they are less sensitive to Fas-mediated apoptosis when compared with control cells. Nevertheless, resistance of LPT cells against apoptosis in Crohn's disease is not limited by the Fas-FasL system, as recent data have shown that T-cells in Crohn's disease grow more rapidly in response to IL-2 than do control cells and are more resistant to IL-2-deprivation-induced apoptosis and nitric oxide-mediated apoptosis (21). This wide resistance to apoptosis is further supported by the fact that inflamed intestinal mucosal T-cells express augmented levels of Bcl-2 and therefore may be resistant to a range of apoptotic mechanisms that involve mitochondrial activity (7).

In view of above mentioned data, the consideration that emerges is that, in the normal (uninflamed) gut mucosa, T-cells exhibit increased spontaneous or activation-induced apoptosis mediated by the Fas pathway that places a limit on the expansion of T-cells following direct and bystander stimulation by specific antigen. On the contrary, in inflamed gut tissue, T-cells are resistant to apoptosis, thereby exhibiting prolonged survival and augmented cytokine production that might, in turn, significantly aggravate the inflammation. Indeed, this defective apoptosis results in inappropriate T-cell accumulation that fosters mucosal inflammation in IBD (22). Resistance of T-cells to apoptotic death can extend their lifespan, and retained activated T-cells in IBD mucosa results in an exceedingly prolonged immune response leading to perpetuation of chronic inflammation. These deleterious events appear to occur particularly in Crohn's disease, as indicated by studies showing that LPT cells have a reduced expression of the proapoptotic Bax protein, while an imbalance between Bax and the antiapoptotic Bcl-2 protein is present in the inflamed mucosa, thereby favoring resistance to apoptosis and contributing to the chronicity of inflammation (9,21-24). Finally, chronic inflammatory epithelial injury can cause tumourigenesis in the gastrointestinal tract, including cancers in the small bowel (Crohn's disease and adenocarcinoma) and colon (IBD and adenocarcinoma) (25). Such resistance to apoptosis also appears to be important in various autoimmune diseases such as the autoimmune lymphoproliferative syndrome. Although a severe defect in Fas-mediated apoptosis in autoimmune lymphoproliferative syndrome can cause certain nongastrointestinal conditions of autoimmunity, the resistance to apoptosis of LPT cells in IBD is not likely to be a principal cause of the disease, but, more likely, is a secondary effect of inflammation that aggravates rather than causes the latter (7).

Considering the above-mentioned data, it is reasonable to assume that eliminating excessive T-cells could restore the intestine to its normal state or, at least, a condition of controlled inflammation (Figures 2,3). Strong evidence for this effect is provided by recent studies in animal models of IBD showing that various antibodies to proinflammatory cytokines and their receptors, such as TNF, IL-12, and IL-6 receptor (IL-6R), appear to suppress chronic intestinal inflammation by the induction of T-cell apoptosis (7,22). A similar effect has been noticed by deletion of $CD44v7^+$ cells (26). Indeed, with two such approaches, the administration of antibodies against IL-

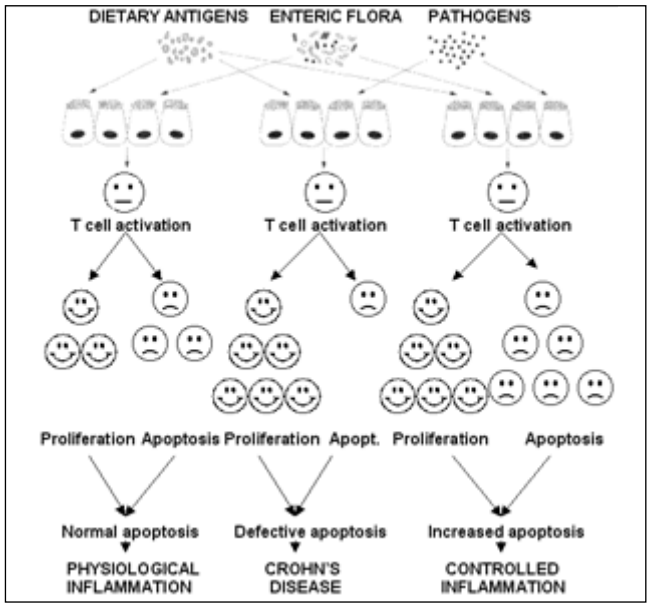

Figure 2) *Different outcomes resulting from the balance between T-cell proliferation and apoptosis (cell death) in the normal gut mucosa, or imbalance during chronic gut inflammation. In health, mucosal T-cell proliferation induced by dietary and enteric flora antigens is counterbalanced by a normal quantity of apoptosis resulting in the low degree of physiological inflammation found in the normal intestine. In Crohn's disease there is augmented T-cell proliferation induced by still undetermined stimuli derived from bacterial antigens, food and possibly unrecognized pathogens. Simultaneously, T-cells die less because of their resistance to apoptosis. This defective apoptosis leads to inappropriate T-cell accumulation that fosters inflammation. If the degree of T-cell apoptosis is augmented in Crohn's disease, an effect apparently mediated, for instance, by anti-tumour necrosis factor-alpha (TNF-α) therapy (infliximab) (22), a new balance is established between the increased rate of proliferation due to inflammation and the increased rate of apoptosis. A condition of controlled inflammation is then established resulting in clinical improvement, and later on decreased cellularity of the gut mucosa (22)*

12 and the IL-6R have been shown to be noticeably effective in both the treatment and prevention of experimental mucosal inflammation. This firstly appeared to be due to the capacity of these antibodies to neutralize key cytokines responsible for the inflammation, either during its inductive phase (anti-IL-12) or its effector phase (anti-IL-6R). Nevertheless, recent reports have disclosed that the mechanisms underlying the therapeutic effects of these antibodies are more profound (7). Both affect the survival of CD4 T-cells mediating the inflammation, such that the major action of the antibodies is on the cells producing the cytokines rather than the cytokines themselves. These observations have significant implications for the design of more effective cytokine-based treatments for IBD and other inflammatory conditions that have a similar pathogenesis (7).

Because the reported antibodies to proinflammatory cytokines appear to suppress chronic intestinal inflammation by induction of T-cell apoptosis, the following recent data concentrates on the role of IL-12 and IL-6 cytokines that suppress the apoptotic process.

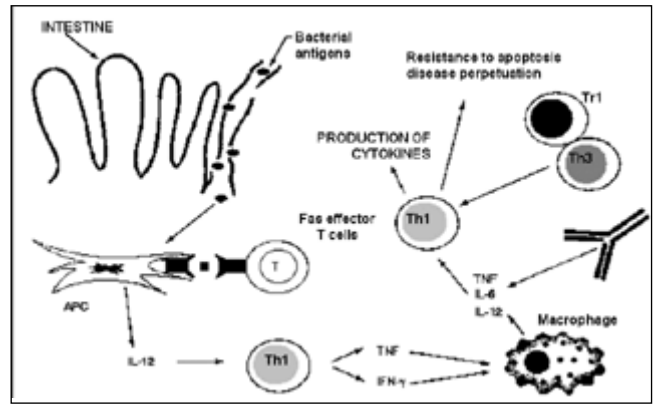

Figure 3) *Resistance of T-cell against apoptosis is a potential key factor in the pathogenesis of inflammatory bowel disease (IBD) (Crohn's disease). In this disease, bacterial antigens in the intestine appear to induce activation of T-cell and T helper 1 (Th1) cell differentiation by interleukin (IL)-12. Tumour necrosis factor (TNF) and interferon (IFN)-γ (mediator substances from Th1 cells) can activate macrophages to release TNF, IL-6 and IL-12, which in turn mediate T-cell resistance against apoptosis in the intestine. This can result in prolonged cytokine production by Fas-expressing Th1 effector cells and, finally, damage of the gut by activation of matrix metalloproteinases. This inflammatory cascade can be arrested by suppressor cytokines released by regulatory T regulatory cells 1 (Tr1) or Th3 cells. Alternatively, it can be suppressed by antibodies such as anti-IL-12 and anti-IL-6R, which induce Th1-cell apoptosis. Therefore, these antibodies alone, or in combination, offer an attractive new approach to the treatment of IBD (7). APC Antigen presenting cell*

IL-12 and apoptosis

In animal (mice) models of trinitrobenzene sulfonic acid-induced colitis, a T helper 1 (Th1)-mediated inflammation replete with cells producing large amounts of IFN-γ, IL-12 and TNF-α, administration of a neutralizing anti-IL-12 antibody is followed after two days by the appearance of apoptotic (terminal deoxynucleotidyltransferase-mediated uridine triphosphate end-labeing positive) CD4+ cells at the site of inflammation in the large intestine. This T cell inducing apoptosis is followed by rapid resolution of the inflammatory process, and might explain the remarkable suppression of intestinal inflammation in various Th1 models of colitis after treatment with anti-IL-12 antibodies. The anti-IL-12-induced apoptosis is a Fas-mediated phenomenon in that the cells being lost are preferentially Fas-bearing cells. Moreover, the therapeutic effect of the antibody is greatly diminished in MRL/lpr mice and cannot mediate apoptosis by the Fas pathway, as well as in normal (SJL/J) mice administered Fas-Fc, an agent that blocks Fas-signalling via FasL, but does not itself signal via Fas. These data strongly suggest that activated Th1 cells producing inflammatory cytokines require the sustained presence of IL-12 if they are to avoid a Fas-mediated apoptotic death. The mechanism by which IL-12 counteracts the Fas pathway in such cells is not yet completely understood, although it is recognized that it does not involve the induction of the anti-apoptotic proteins, Bcl-2 or Bcl-x_L, and therefore may act on mitochondria-independent DISC signalling events (Figure 1) (7).

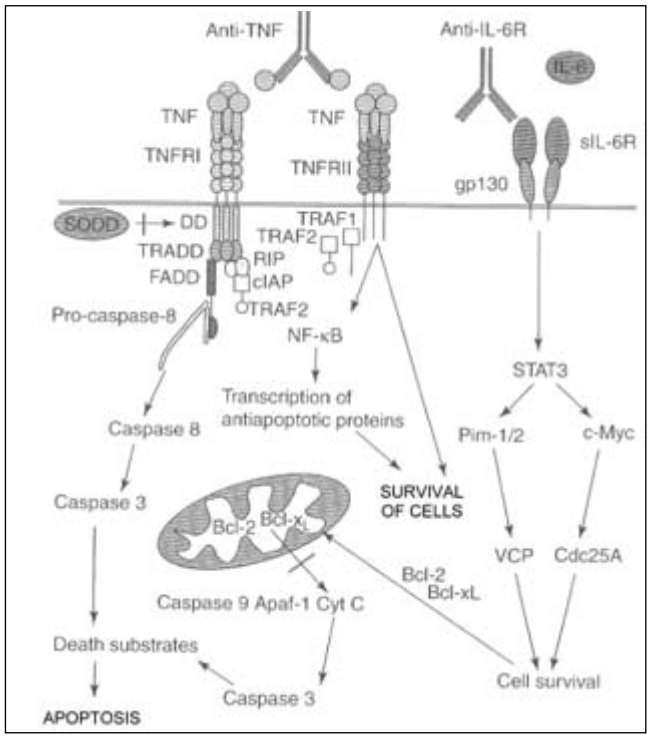

Figure 4) *Prevention of T-cell apoptosis in chronic gut inflammation via interleukin (IL)-6 trans-signalling and tumour necrosis factor (TNF) signalling events. Chronic gut inflammation is associated with increased production of both IL-6 and TNF. IL-6 can bind to IL-6R lamina propria T-cells (LPT) upon binding to its soluble receptor (sIL-6R) and the gp 130 molecule. Signalling by gp 130 leads to activation of signal transducer and activator of transcriptor 3 (STAT3) and STAT3 target molecules such as Pim-1/2, c-myc and lastly Bcl-2 and Bcl-x_L. This pathway induces increased cell survival and T-cell resistance against apoptosis in inflammatory bowel disease (IBD) patients. On the other hand, blockade of IL-6 trans-signalling causes remission of gut inflammation by suppression of T-cell resistance against apoptosis. Equally to IL-6, TNF can cause increased cell survival by signalling via the tumour necrosis factor receptor (TNFR) I/II and activation of nuclear factor (NF)-κB, even though activation of TNFR-I can also induce apoptosis by activation of caspase 6. Nevertheless, recent data indicate that antibodies to TNF (which are clinically effective in inducing rapid suppression of gut inflammation in IBD patients) (42) seem to mediate their effects at least in part by rapid induction of T-cell apoptosis in the inflamed intestine, signifying an important function for TNF in regulating gut T-cell survival in IBD patients (7). Apaf-1 Apoptotic protease activation factor; cIAP Cellular inhibitor-of-apoptosis proteins; Cyt c Cytochrome c; DD Death domain; FADD Fas- associated protein with death domain; RIP Receptor-interacting protein; SODD Silencer of death domains; TRADD TNFR-I-associated death domain protein; TRAF TNF receptor-associated factor; VCP Valosine-containing protein*

IL-6 and apoptosis

Some data indicate a direct pathogenic role for the complex of IL-6 and its soluble receptor (sIL-6R) in IBD (Crohn's disease), thereby suggesting a therapeutic potential of disrupting this form of cytokine signalling. It has been demonstrated that LP macrophages release sIL-6R, which may complex with IL-6 and stimulate gp 130 on the surface of intestinal LPT cells, leading to a signal transducer and activator of transcription (STAT)-3 dependent activation of a cascade of antiapoptotic genes such as *Bcl-2* and *Bcl-x_L*, and then to apoptosis resistance (27). By blocking the IL-6 trans-signalling pathway, restoration of T-cell susceptibility to apoptosis leads to suppression of Th1-mediated colitis in several animal models of chronic intestinal inflammation, including colitis related to IL-10 deficiency, trinitrobenzene sulfonic acid-induced colitis, and colitis occurring in severe combined immunodeficiency mice upon transfer of naive (CD62L+, CD45Rb^{hi}) T-cells (7,27). Support for such IL-6 trans-signaling comes from the information that a gp130-Fc fusion protein that interacts with sIL-6R but not with membrane-bound IL-6R could also ameliorate various forms of colitis, presumably by blocking the interaction of the complex with membrane-bound IL-6R and thus the anti-apoptotic IL-6 signal. Furthermore, it has been shown in patients with Crohn's disease (a Th1-mediated inflammation in humans) that, while T-cells from the LP lack IL-6R, they appear to have transduced an IL-6-specific signal in vivo in which they contain activated STAT3. Additionally, IL-6-sIL-6R complexes have been observed in the serum of patients with Crohn's disease, and treatment of Crohn's disease LPT cells with anti-IL-6R results in both the disappearance of cells containing activated STAT3 and augmented cell apoptosis (Figure 4) (7). In contrast to the antiapoptotic effect mediated by IL-12, the antiapoptotic effect mediated by IL-6 trans-signalling, may involve Bcl-2 and Bcl-x_L. Indeed, these proteins are increased in LP cells from patients with Crohn's disease leading to an increase of the Bcl-2/Bax ratio and are known to be induced by STAT3. In view of these new data involving IL-6R signalling, the reduced apoptosis in the inflammatory milieu of the inflamed gastrointestinal tract can be attributed to the fact that in this milieu the cells are subject to IL-6R trans-signalling and the induction of antiapoptotic proteins (7).

Specific features of the apoptotic process in ulcerative colitis and Crohn's disease patients have been described. Delayed neutrophil apoptosis is a feature of persistent acute inflammation and neutrophil-mediated damage has been shown to be associated with the development of IBD. Neutrophils isolated from patients with IBD have shown reduced spontaneous apoptosis compared with those of cancer patients. Mesenteric venous serum of IBD patients contributed to this delay, which contained higher levels of IL-8. Furthermore, procaspase 3 expression was also decreased in IBD neutrophils, contributing to reduced spontaneous and Fas antibody-induced apoptosis. Therefore, neutrophil apoptosis may be altered in Crohn's disease and ulcerative colitis through the release of antiapoptotic cytokines and altered caspase expression (28-30). Thus, alterations in cell death mechanisms may lead to persistence of the inflammatory response associated with IBD (30).

It has been shown that FasL mRNA is strongly expressed in active ulcerative colitis lesions, but not in those with active proctitis-type ulcerative colitis and/or active Crohn's disease (31). The FasL mRNA positive cells infiltrating ulcerative colitis lesions are largely CD3 T lymphocytes. Therefore, the CD3 T lymphocytes with surface FasL may be involved, at least in part, in the pathogenesis of this disease. The high expression of FasL in ulcerative colitis may induce IL-8 production and secretion from colonic epithelia, promoting the migration and activation of lymphocytes and neutrophils. As a consequence, the increase in FasL on CD3 T lymphocytes is thought to play a role in the chronic inflammation, directly through the induction of apoptosis in Fas-expressing colon epithelia, and indirectly through IL-8-mediated migration and

activation of neutrophils and lymphocytes, leading to the progression of mucosal lesions (31). Collectively, these data indicate that the increased FasL on CD3 T lymphocytes results in the progression of mucosal lesions in ulcerative colitis, with the exception of the proctitis type. Of the several types of ulcerative colitis known, the proctitis type has unique features, with involvement limited to the rectum, a milder clinical course and a better prognosis than other types. These unique manifestations indicate that its pathogenesis may differ from that of other types of ulcerative colitis. Because the FasL transcripts are not increased in active lesions of proctitis-type ulcerative colitis, Fas-FasL mediated apoptosis is not involved in the pathogenesis of this type of disease (31). From a pathogenic viewpoint, in ulcerative colitis, soluble CD95L-mediated epithelial apoptosis may lead to a breakdown of the epithelial barrier function facilitating the invasion of pathogenic microorganisms (32). In active Crohn's disease lesions, macrophages, but not CTLs, may be critically implicated in the onset and/or progression of Crohn's disease, thereby explaining the normal FasL mRNA expression at least in some Crohn's disease patients (31).

Granzyme B mRNA is elevated, and its levels correlate with IFN-γ mRNA levels in Crohn's disease at first presentation, but not in ulcerative colitis or control mucosal biopsies. Granzyme B is expressed in CD3+ and CD8+ T-cells, and, in addition, there are significantly more apoptotic cells in the LP of a Crohn's disease patient's mucosa. Collectively, these data suggest that granzyme B-expressing T lymphocytes are present in the focal mucosal lesions of Crohn's disease, together with spatially related apoptotic cell death, thereby supporting the hypothesis that T-cell-mediated cytotoxic effector mechanisms may play a role in Crohn's disease (33). From another viewpoint, and compared with controls, Crohn's disease mucosa appears to contain similar numbers of Bcl-2+, but fewer Bax+ cells, while ulcerative colitis mucosa contains fewer Bcl-2+, but more Bax+ cells; thereby, the Bcl-2/Bax ratio is significantly higher in Crohn's disease and lower in ulcerative colitis (24). These results indicate that Crohn's disease may represent a disorder where the rate of T-cell proliferation exceeds that of cell death, and insufficient T-cell apoptosis may interfere with clonal deletion and maintenance of tolerance, thereby resulting in inappropriate T-cell accumulation contributing to chronic inflammation (21) and potential progression to cancer development. However, recent data indicate the existence of equal levels of expression of Bax protein and mRNA in the epithelia of the normal colon and inactive ulcerative colitis, whereas the levels of expression of Bax protein and mRNA are markedly reduced in inflamed ulcerative colitis colonic epithelium (34). As a consequence, the expected prolonged apoptotic process, due to defective expression of the proapoptotic Bax protein in active ulcerative colitis, may also contribute to chronic inflammation and potential progression to tumourigenesis.

IBD-apoptosis-carcinogenesis

IBD, particularly ulcerative colitis, is associated with an increased incidence of neoplastic transformation (35). Most studies have agreed that the risk of cancer in patients with ulcerative colitis begins with a disease duration of seven to 10 years and rises about 10% per decade, reaching approximately 30% at 25 years. Those at greatest risk are patients with extensive colitis, defined as contiguous disease from the rectum beyond the splenic flexure or pancolitis. Early age-at-onset of colitis also appears to be associated with an increased cancer risk. The increased risk for colorectal carcinoma in patients with Crohn's disease has been reported to be as much as four to 20 times that of the general population (36). High levels of proinflammatory leukotrienes (LTs) and upregulated expression of cyclooxygenase-2 are characteristic of inflammation and have been implicated in cell survival and early colon carcinogenesis. In particular, LTs cause a time- and dose-dependent increase in expression and/or membrane accumulation of cyclooxygenase-2, beta-catenin, and Bcl-2, as well as prostaglandin E2 production, and the effects of LTs on these transformation-associated proteins correlate well with the ability of LTs to reduce programmed cell death (35). Therefore, these observations suggest that IBD is associated with the expression and distribution of proteins that are characteristic of transformed cells and such pathological conditions may involve a signalling mechanism comprising an altered rate of apoptosis.

To throw light on accelerated epithelial cell turnover as an important risk factor of dysplasia and carcinoma development in patients with long standing ulcerative colitis, some studies have shown that the numbers of mitoses, apoptotic bodies, Ki-67-immunoreactive cells, and p21 WAF1/CIP1-immunoreactive cells per 1000 crypt cells, as well as the numbers of p53-positive cells per crypt are significantly higher in active disease than in either remitting ulcerative colitis cases or normal cases. In addition, the mitotic, apoptotic, and Ki-67 labelling indices are increased in remitting disease of more than 10 years' duration, in comparison with those of less than 10 years' duration or the normal group (37). Therefore, the accelerated epithelial cell turnover caused by chronic inflammation and epithelial damage may predispose the mucosa to DNA damage, resulting in an increased risk of mutation in line with dysplasia and carcinoma development in IBD patients (37).

The combination of the above-mentioned data may lead to the consideration that the increased cell proliferation, as indicated by the elevated expression of oncogene Ki-67, and the defective apoptosis represent two risk factors for cancer development in IBD. It is also known that the progressive accumulation of genetic changes in both oncogenes and tumour-suppresor genes parallels the clinical and histopathological progression from normal colonic epithelium through benign adenomas to dysplasia and frank colon cancer (38).

Contrary to the data of other countries, the incidence of cancer in Greek patients with IBD appears to be low (39,40), although as yet there is no possible explanation for this observation. However, in a recent pilot study involving 22 Greek patients (16 with Crohn's disease and 6 with ulcerative colitis), an increased mucosal expression of Bax protein was noticed in 50% to 62% of the patients. Moreover, an elevated expression of oncogene Ki-67 was also observed in three quarters of the patients (41). These preliminary results suggest a possible existence of a balance between cell proliferation (indicated by Ki-67 overexpression) and apoptosis (indicated by Bax protein overexpression), thereby reflecting no defective apoptotic process that may explain, at least in part, the low incidence of cancer development in Greek IBD patients.

CLOSING COMMENTS

Apoptosis of LPTs seems to be necessary to maintain immune homeostasis in the specialized microenvironment of the gut mucosa by downregulating the gut immune response and elim-

inating reactive clones. Whereas normal LPTs manifest an increased susceptibility to Fas-mediated apoptosis, IBD is associated with the resistance of LPTs to multiple apoptotic pathways. Such defective apoptosis may interfere with clonal deletion and maintenance of tolerance that may be a key factor for inappropriate T-cell accumulation contributing to chronic mucosal inflammation in IBD. Moreover, abnormal regulation of apoptosis results in disease, including cancer (24). Novel therapeutic strategies such as anti-IL-6R and anti-IL-12 antibodies appear to mediate their rapid beneficial effects on colitis activity at least in part by the suppression of T-cell resistance against apoptosis and the consecutive induction of mucosal T-cell death resulting in the limited expansion of activated T-cells and deletion of autoreactive T-cell clones. In addition, anti-TNF-α antibodies that are currently used with considerable success in the treatment of Crohn's disease have also been suggested to act by inducing rapid and specific increase in apoptosis of T-cells in the gut mucosa, possibly altering (increasing) the Bax/Bcl-2 ratio (42). It should be noted that the infiximab (anti-TNF-α antibodies) therapy is based on the consideration that Crohn's disease is mainly a Th1-related inflammatory condition, in which cytokine profiles of the Th1 variety (including TNF-α) have been particularly implicated in the pathogenesis of the tissue damage. On the other hand, ulcerative colitis is probably a Th2-related inflammation. Although therapy with anti-IL-6R or anti-IL-12 antibodies also induces death of LP Th1 T-cells, it is not clear whether such treatment would also induce cell death of T regulatory 1 or Th3 cells; an event that would question the long-term beneficial effects of this therapeutic strategy. However, several findings indicate that Th3 and T regulatory 1 cells are not subject to anti-IL-12-mediated apoptosis. Indeed, these cells are not Th1 cells and thus lack a fully competent IL-12 receptor. Moreover, there is good evidence that transforming growth factor-beta extinguishes IL-12Rβ2 chain expression so that Th3 cells would primarily downregulate IL- 12R expression, and lastly, administration of anti-IL-12 to mice has been shown to increase transforming growth factor-beta and IL-10 secretion (7).

The experimental observations on anti-IL-6R or anti-IL-12 therapeutic approaches are relevant to human IBD, and to other human Th1- or Th2-mediated inflammatory conditions. Therefore, in Th1-mediated inflammation such as Crohn's disease, the supply of an IL-12 inhibitor such as anti-IL-12 will not only lead to resolution of disease by neutralization of the cytokines that initiate the Th1 pathway of inflammation, but also by the death of induced CD4+ Th1 cells. A similar effect can be obtained by the provision of an IL-6 inhibitor such as anti-IL-6R because such treatment also results in both neutralization of cytokines and cell death. In this case, however, the therapy is probably not limited to Th1-mediated inflammation because it applies to end-stage CD4+ effector T-cells arising from any T-cell differentiation pathway. Thus, anti-IL-6R antibodies may be used in ulcerative colitis, more like a Th2-mediated than a Th1-mediated inflammation, in that neither IL-12 nor IFN-γ secretion is increased by isolated LP macrophages or T cells, respectively, and Th2 T cell-mediated animal models of inflammation resemble ulcerative colitis pathologically (7).

The potential relevance of modulating T-cell resistance against apoptosis as a therapeutic strategy in IBD is underlined by the recent observation that drugs that facilitate Fas-mediated apoptosis can be used for suppression of T-cell-mediated autoimmune disease. However, the T-cell death brought about by anti-IL-12 and anti-IL-6R antibodies in intestinal inflammation is likely to occur via largely independent mechanisms. This introduces the possibility of inducing death of inflammatory CD4+ T-cells in independent and possibly synergistic ways. Therefore, the future of therapeutic strategy of IBD might lie in combined anticytokine therapy rather than therapy with a single agent.

Such therapeutic approaches, by inducing T-cell apoptosis, may alter the potential for tumourigenesis observed in IBD. Moreover, future investigation of genetic alterations of oncogenes involved in apoptosis may identify specific population groups associated with a high or low (observed in a small number of Greek patients) risk of cancer development in IBD.

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