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Chapter 2.

Apoptosis as a therapeutic paradigm in inflammatory bowel diseases

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Abstract

Evidence is increasing that a defect in apoptosis is involved in the pathogenesis of inflammatory bowel diseases (IBD), including Crohn’s disease (CD) and ulcerative colitis (UC). CD seems to be the cause of an intrinsic defect in the apoptotic pathway of (autoreactive) T cells, resulting in excessive T cell responses. In UC, an increased rate of apoptosis of epithelial cells is observed. In this review we will describe apoptotic mechanisms and their association to IBD. In addition, we will review how specific therapeutic approaches interact at different levels with the apoptotic pathway.
Introduction

Inflammatory bowel diseases (IBD), including Crohn’s disease (CD) and ulcerative colitis (UC) are chronic inflammatory disorders of the intestinal tract with an unknown aetiology. Although the pathogenesis of IBD is not well understood, evidence is increasing that excessive mucosal T cell-dependent responses in IBD are due to a defect in apoptosis. Muramyl dipeptide stimulated monocyte-derived dendritic cells from CD carriers of double-dose NOD2 mutations demonstrated a change in gene expression profiles resulting in a negative regulation of apoptosis.\(^1\)

Apoptosis or programmed cell death is a normal physiological process, which plays an important role in the development and morphogenesis, cellular homeostasis and the deletion of damaged and autoreactive cells, e.g. the formation of the digits,\(^2\) the development of the brain,\(^3\) and reproductive organs,\(^4\) the negative selection of T cells in the thymus,\(^5\) and the deletion of infected and cancer cells.\(^6\) In the gut, apoptosis is important in the homeostasis of the epithelium by regulating cell numbers of epithelial cells and in the elimination of lamina propria T lymphocytes (LPLs) to prevent an excessive immune response because of the constitutive examination of luminal antigens.\(^7,8\)

In contrast to necrosis, apoptosis will not lead to an inflammatory reaction, because of a tightly and controlled process in which the cell shrinks, the chromatin condensates and marginates at the nuclear membrane and ‘budding’ of the plasma membrane will result in apoptotic bodies that contain cytoplasm and organelles, which can be phagocytosed by immune cells.\(^9\) Necrotic cells, however, are swollen and leaky and finally, because of cellular and nuclear lysis, the complete contents of the cells will be released in the surrounding tissues, resulting in inflammatory reactions.\(^10\)
The cell cycle

The cell cycle is regulated by a large family of enzymes mentioned as cyclin-dependent kinases (CDKs), which play a key role in the phosphorylation and consequently activation of downstream molecules that are involved in DNA replication and mitosis (see figure 1). CDKs are activated by association with cyclins and are inhibited by other kinases and phosphatases such as p15, p16, p21 and p27. In a controlled cell cycle, the interaction between CDKs, cyclins and CDK inhibitors is tightly regulated to induce cell growth when necessary, but to prevent excessive cell growth that could result in tumourgenesis. The cell cycle suppressor proteins p15 and p27 could be activated by transforming growth factor (TGF)-β, which plays an important role in the inhibition of epithelial cell proliferation 11,12.

It has been shown that TGF-β expression is increased in murine LPLs and colonic epithelial cells and that epithelial apoptosis is increased in mice or rats that developed colitis by oral administration of dextran sodium sulphate (DSS) 13,14. These results indicate that epithelial cells and lymphocytes in UC becomes more sensitive to apoptosis by a constitutively inhibition of cell growth. In human UC patients, however, the results are not so clear. Different studies have demonstrated that apoptosis of LPLs in both in UC as well as CD patients is decreased 7,15,16. On the contrary, several other studies have shown that LPLs of UC patients have a delayed cell cycle progression leading to increased apoptosis of these lymphocytes 17-19. Moreover, apoptosis of epithelial cells, mainly located at the apical surface of the mucosa is increased in UC patients compared to normal controls 17,20. It could be hypothesized that increased apoptosis of epithelial cells increases mucosal exposure to intestinal bacteria inducing an inflammatory response in UC patients. This hypothesis is still speculative, but the treatment of IL-10 deficient mice with the non-steroidal anti-inflammatory drug piroxicam has been shown to enhance apoptosis of epithelial cells in combination with a decreased barrier function and acceleration of the development of colitis 21.

In response to cellular stress, e.g. DNA damage and hypoxia, the tumour suppressor gene p53 becomes activated by phosphorylation resulting in the activation of the CDK inhibitor p21, which will finally lead to apoptosis 22. Stabilisation and activation of
p53 is initiated by ARF, which blocks the Mdm2-mediated suppression of p53. Mdm-2 mediates ubiquitination of p53, which will be degenerated by the proteasome. Binding of ARF inhibits the ubiquitin-ligase activity of Mdm-2, thereby increasing active p53 levels. ARF expression is mediated by the transcription factor E2F, which is activated by several oncogenes, e.g. ras and myc, and inhibited by the tumour suppressor gene product retinoblastoma (Rb). It has been demonstrated that LPLs of CD patients have an increased expression of phosphorylated Rb and a decreased expression of phosphorylated p53, both resulting in resistance to apoptosis and dynamic cellular expansion. On the contrary, p53 is overexpressed in areas of acute inflammation in UC patients.
Figure 1. The cell cycle. The cell cycle is tightly regulated by CDKs, which are activated by association with cyclins and are inhibited by p15, p16, p21 and p27. TGF-β is a suppressor of p15 and p27 and consequently inhibits epithelial cell proliferation. In response to cellular stress, p53 becomes activated resulting in the activation of the CDK inhibitor p21, the activation of the pro-apoptotic proteins Bax and Apaf-1 and the inhibition of the anti-apoptotic proteins Bcl-2 and Bcl-X<sub>L</sub>, finally leading to apoptosis. Mdm-2 mediates ubiquitination of p53 so that it will be degenerated by the proteasome. ARF is able to stabilise and activate p53 by blocking the Mdm2-mediated suppression of p53. The expression of ARF is mediated by E2F which is activated by several oncogenes, including ras and myc and is inhibited by the tumour suppressor product Rb. Oncogenes also activate Akt leading to Mdm2 activation.
Death receptor pathway

Both internal as well as external stimuli are able to induce apoptosis, such as the activation of different surface molecules, DNA damage caused by defective DNA repair mechanisms and irradiation, an uncontrolled cell cycle, cytotoxic drugs or a lack of survival signals (see figure 2). Three major pathways in the initiation of apoptosis have been described: 1) the death receptor pathway, 2) the mitochondrial pathway and 3) the endoplasmatic reticulum stress pathway. The death receptor pathway is an extrinsic apoptosis pathway characterised by the interaction between death receptors and their ligands, the recruitment of adapter proteins and the activation of several caspases. This classical pathway which is mainly caspase-dependent is restricted to the so-called type I cells 27. Death receptors belong to a family of tumour necrosis factor receptors (TNFRs) and nerve growth factor receptors (NGFRs) of which the Fas- (CD95) and TNFR-mediated apoptosis are the best studied. Fas is a type I transmembrane receptor that is widely expressed and constitutively expressed by T lymphocytes, whereas FasL is a type II transmembrane protein that is induced on activated T lymphocytes. Upon ligation of FasL, Fas forms trimers and through its death domain (DD) recruitment of the adapter molecule Fas-associated death domain (FADD) is achieved. FADD contains death effector domains (DEDs) that recruit and binds to procaspase-8 (flice) to form the death-inducing signalling complex (DISC) 28. Pro-caspase-8 is activated by autoproteolytic cleavage of caspase-8, which consists of two heterodimers of two small and two large subunits 29. Active caspase-8 cleaves downstream effector procaspases in active caspases that cleave several transcription factors, structural proteins and enzymes involved in DNA repair and cleavage and cell cycle progression, finally resulting in cell death. Neutrophils isolated from both CD as well as UC patients exhibit a decreased expression of pro-caspase-3, one of the substrates of caspase 8, leading to delayed apoptosis 30. The Fas-mediated apoptosis can be inhibited by Fas-associate-death domain-like interleukin-1β-converting enzyme (Flice)-inhibitory protein (Flip), which is an intracellular protein that prevents the cleavage of pro-caspase-8 31,32. The expression of Flip is enhanced by LPLs from CD compared to normal controls, resulting in resistance to apoptosis 19,33. In addition, in CD patients the expression of Fas is reduced in T cells and macrophages located in the LP in situ, whereas rates of FasL-expressing cells in the LP are increased,
indicating that LPLs of CD patients are less sensitive to extrinsic apoptotic signals. Isolated LPLs from CD patients that were cultured with a CD2 activation pathway stimulus express similar levels of Fas compared to controls; however they are less sensitive to Fas-mediated apoptosis induced by Fas antigen cross linking. Also in UC there are indications that the Fas-ligand mediated apoptosis is disturbed in LPLs, although some of the results are contradictive. Suzuki et al. have shown that the expression of FasL is increased by memory CD4+ LPLs, but that the expression of Fas is not different compared to normal controls. Since the apoptotic cell ratio induced by anti-Fas antibody did not change, it seems that memory CD4+ LPLs are less sensitive to Fas-ligand mediated apoptosis in UC patients. However, Strater et al. have demonstrated that in UC patients, the expression of both FasL as well as Fas is increased by LPLs, indicating that LPLs of UC patients are more sensitive to apoptosis. Moreover, they have shown that also colonic epithelial cells have an increased sensitivity to apoptosis. Besides, Yukawa et al. have demonstrated that in active UC, an increased number of FasL-expressing cells was present in the mucosa and that the number of apoptotic cells was increased compared to UC patients in remission, non-IBD colitis and controls. In conclusion, LPLs of CD patients are less sensitive to Fas-mediated apoptosis, whereas Fas-mediated apoptosis sensitivity seems to be increased in LPLs and epithelial cells of UC patients.

Besides Fas, apoptosis can also be induced through the TNFRs upon binding to its ligand TNF-α. TNFRs are type I transmembrane proteins of which only TNFR-I contains a DD. Since TNFR-II lacks a DD, this molecule is mainly involved the survival of the cell, whereas TNFR-I activates both cell survival pathways as well as death signalling. Upon ligation with TNF-α, TNFR-I forms trimers via its DD, followed by the recruitment of the adapter molecule TNFR-associated DD (TRADD) and FADD. FADD recruits pro-caspase-8 and activation of this molecule by dimerisation results in the cleavage of downstream effector caspases and finally in apoptosis, a pathway similar as described for Fas-mediated apoptosis. Conversely, FADD is able to activate cell survival pathways as well by the recruitment of TRAF-2 and RIP, molecules that induce pathways leading to mitogen-activated protein kinase (MAPK) and NF-κB, respectively. NF-κB is a transcription factor that induces the expression of pro-inflammatory genes, including TNF-α, IL-1β, IL-6 and
IL-12, and anti-apoptotic genes such as Flip, Traf-1, Bcl-2 and Bcl-xl (see below). Bregenholt et al. demonstrated that apoptosis of LPLs is mainly induced via the Fas-mediated pathway and not via the TNF-α receptor in CD4⁺-transferred SCID mice with colitis 35.

Since TNFR-II does not harbour a DD, they are not able to induce apoptosis via FADD, but there are some data that also TNFR-II can induce TNF-α-mediated apoptosis 36-39. Nevertheless, over-expression of TNFR-II promotes colitis in SCID mice transferred with CD4⁺CD62L⁺ T cells characterised by aggravated Th1 response and apoptotic resistance, indicating that TNFR-II plays a prominent role in cell survival 40. In addition, it has been shown that TNFR-II expression by LPLs and peripheral blood T lymphocytes is increased in CD patients, whereas TNFR-I levels were similar to normal controls 40.
Figure 2. Apoptotic signalling. The death receptor pathway is characterised by the activation of Fas by FasL resulting in the recruitment of FADD and pro-caspase-8 to form the death-inducing signalling complex (DISC). Pro-caspase is cleaved in caspase-8, which cleaves other downstream pro-caspases into their active form, including pro-caspase-3, leading to apoptosis. Apoptosis could be prevented byFlip, since it inhibits the cleavage of pro-caspase-8. This pathway can be amplified through the mitochondrial pathway by the cleavage of Bid by caspase-8. Also stimuli such as DNA damage are able to activate the mitochondrial pathway. DNA damage leads to the activation of p53 and consequently of the activation of pro-apoptotic proteins Bak and Bax. Overexpression of Bcl-2 and Bcl-X₇ prevents activation of Bak and Bax, resulting in cell survival. Bid, Bak and Bax contribute to permeabilisation of the outer membrane or by interaction with voltage dependent anion channels in the outer membrane mitochondrion, leading to the release of cytochrome c in the cytoplasm. Cytochrome c forms together with Apaf-1 and pro-caspase-9 the apoptosome resulting in activated caspase-9, which cleaves pro-caspase-3 into its active form, leading to downstream activation of other caspases and finally in apoptosis. The apoptosome and caspase-3 could be inhibited by IAPs, so that apoptosis is prevented.
Type II cells are not able to induce apoptosis though the classical pathway since procaspase-8 concentrations are too low to induce a strong enough caspase signalling cascade. Therefore the signal has to be amplified through the mitochondrial-mediated pathway, which is activated by the cleavage of Bid by caspase-8. Truncated Bid is capable to translocate from the cytoplasm into the mitochondria where it induces conformational changes in the pro-apoptotic protein Bax, resulting in the release of cytochrome c in the cytoplasm. Cytoplasmic cytochrome c interacts with the adaptor molecule protease-activating factor (Apaf)-1 in a dATP-dependent manner to assembly the apoptosome that initiates the activation of pro-caspase-9. Activated caspase-9 cleaves other downstream caspases, including caspase-3, -7 and -6, consequently inducing apoptosis.

In addition to activation via the classical pathway, other stimuli such as DNA damage caused by e.g. irradiation, chemotherapeutic drugs, stress molecules and a lack of growth factors also can promote mitochondrial-mediated apoptosis. A central role in the mitochondrial-mediated apoptosis plays the Bcl-2 family of proteins, which harbours both pro- as well as anti-apoptotic members. Dependent on the presence of the highly conserved Bcl-2 homology domains (BH1 to BH4), the Bcl-2 family of proteins can be defined into three groups. The first group is characterised by the presence of all the four domains and by their anti-apoptotic effects (e.g. Bcl-2 and Bcl-XL). The second and third group, however, both consist of proteins with pro-apoptotic effects and are distinguished by the presence of the domains BH1 to BH3 (e.g. Bax, Bak) or only the BH3 domain, respectively (e.g. Bid). Activation of p53 e.g. due to DNA damage, Bax and Bak are activated, which are thought to contribute to the permeabilisation of the outer membrane of the mitochondrion \(^{41}\) or by the interaction with voltage dependent anion channels in the outer membrane \(^{42}\), leading to the release of cytochrome c in the cytoplasm and consequently to apoptosis of the cell. Overexpression of Bcl-2 and Bcl-XL prevents oligomerisation and activation of Bak and Bax, which will result in cell survival \(^{43}\). In healthy colonic tissue of mice it has been shown that the expression of Bcl-2 is prominent in the base of the crypts, whereas surface epithelial cells express increased levels of Bax, consistent with cell growth in the bottom of
the crypts and cell death at the surface \(^{44}\). However, in both mice and humans, it has been demonstrated that different components of the mitochondrial pathway are affected in colitis. Go\(_{i2}\)-deficient mice, which spontaneously develop an UC-like disease, exhibit an increased apoptosis of lymphocytes associated with decreased levels of Bcl-2 leading to regression of Peyer’s patches, both in number as well as in size \(^{45}\). Besides, mice which are deficient for poly-(ADP-ribose) polymerase-1 (PARP-1), which is activated in response to DNA damage, develop less severe TNBS-induced colitis accompanied with an increased expression of Bcl-2 and reduced apoptosis of epithelial cells compared to wild type mice \(^{46}\). TNBS-induced colitis in wild type mice results in a decrease of the anti-apoptotic protein Bcl-2, whereas the pro-apoptotic protein Bax remains unchanged \(^{46}\). This indicates that disruption of the epithelial barrier due to a genetic defect or a reagent results in apoptosis of LPLs and epithelial cells. In human CD patients, however, a decreased Bax to Bcl-2 ratio accompanied by a decreased level of Bax and/or an increased level of Bcl-2, results in apoptotic resistance of mucosal T lymphocytes \(^{34,47,48}\).
Apoptosis as a therapeutic paradigm in IBD

Currently, (gluco-) corticosteroids, thiopurines, methotrexate, 5-amino salicylic acid (5-ASA) and its derivates and inhibitors of TNF-α are commonly used in IBD. Several of these compounds interfere at different levels with the apoptotic pathway. Sulphasalazine is a conjugate of 5-ASA and sulphapyridine and has been shown to inhibit the phosphorylation of NF-κB directly \(^{49}\). Since NF-κB is not only a transcription factor for several pro-inflammatory genes, but also for anti-apoptotic genes, the inhibition of NF-κB may lead to an increased susceptibility to apoptosis. Doering et al. demonstrated that sulphasalazine, however not 5-ASA and sulphapyridine alone, induces apoptosis of LPLs in a Fas-independent way \(^{16}\). Sulphasalazine treatment of Jurkat T cells results in a down-regulation of the anti-apoptotic molecules Bcl-2 and Bcl-X\(_L\) and subsequent activation of caspase-3 and -9, indicating that sulphasalazine acts via the mitochondrial pathway to induce apoptosis \(^{16}\). Another common used drug in the treatment of IBD is azathioprine. Tiede et al. have demonstrated that azathioprine and its metabolites 6-mercaptopurine and 6-thioguanine nucleotides only induce apoptosis of CD4\(^+\) lymphocytes when they are co-stimulated through CD28 \(^{50}\), suggesting that azathioprine induces apoptosis of activated T lymphocytes. The TNF-α neutralising drugs infliximab and etanercept are both efficacious in the treatment of rheumatoid arthritis, however; only infliximab has been shown to be effective in the treatment of CD \(^{51,52}\). This suggests different mechanisms in which both TNF-α neutralizing drugs act. Van den Brande et al. have demonstrated that both infliximab as well as etanercept neutralize soluble TNF-α effectively \(^{53}\). However, Infliximab but not etanercept has the capacity to induce apoptosis of activated peripheral blood lymphocytes, LPLs and monocytes \(^{53,54}\). Apoptosis induced by infliximab is associated with an increase in the Bax/Bcl-2 ratio in Jurkat T cells that are CD3/CD28 stimulated, but not in unstimulated cells \(^{54}\).

Also potential new therapeutic strategies in IBD correlate with apoptosis. IL-6 seems to play an important role in the maintenance of the intestinal inflammation \(^{55}\). The expression of IL-6 and its soluble receptor by LPLs is increased in IBD patients \(^{55}\) and will
result to an elevated expression of STAT-3 and its nuclear translocation leading to the transcription of anti-apoptotic genes, including Bcl-2 and Bcl-xl. Elevated IL-6 expression will finally lead to an increased resistance to apoptosis of LPLs and is therefore an interesting target in the treatment of IBD. Both antibodies against the IL-6 receptor as well as the soluble form have been shown to reduce inflammation processes in several experimental colitis models \textsuperscript{50,56,57}. Treatment with antibodies against the IL-6 receptors suppresses the mRNA expression of adhesion molecules such as ICAM-1 and VCAM-1 and pro-inflammatory cytokines including IFN-\(\gamma\), TNF-\(\alpha\) and IL-1\(\beta\) \textsuperscript{56}. Moreover, the expression of active STAT-3 is reduced, whereas the number of apoptotic LPLs is increased \textsuperscript{57}. In addition, \textit{in vitro} studies showed that LPLs of CD patients undergo apoptosis after being treated by antibodies against the IL-6 receptor \textsuperscript{50}. Antibodies against the Th1-inducing cytokine IL-12 also reduce experimental colitis and induce apoptosis of LPLs in a Fas-dependent way, because Fas-deficient or Fas-Fc treated mice are resistant to anti-IL-12 treatment \textsuperscript{58}.

NF-\(\kappa\)B activation is observed in IBD, and a potential new strategy would be to block this transcription factor by NF-\(\kappa\)B decoy oligonucleotides \textsuperscript{59}. These decoy oligonucleotides induces CD4\(^+\) T lymphocyte apoptosis and reduces the inflammation in both the Th1-mediated TNBS-induced colitis model as well as the Th2-mediated oxazolone-induced colitis \textsuperscript{59}. Also the use of antisense oligonucleotides to inhibit the anti-apoptotic protein Flip restores apoptosis in isolated LPLs \textsuperscript{33}.

A second approach in the treatment of IBD is the use of plant-derived compounds that harbour anti-inflammatory and anti-oxidant effects. Garlicin is a compound extracted from garlic corn and has been shown to be effective in TNBS-induced colitis in rats by reducing the expression of the anti-apoptotic protein Bcl-2 by lymphocytes resulting in increased apoptosis \textsuperscript{60}. Also resveratrol, which is a polyphenolic compound derived from grapes and wine, is able to reduce TNBS-induced colitis in rats and to enhance apoptosis \textsuperscript{61}. The mechanisms by which these compounds are capable in reducing intestinal inflammation and enhancing apoptosis have still to be elucidated.
Finally, it is also possible to make use of immunosuppressive capacities of several micro-organisms to escape the immune system of the host. Ga\(_2\)-deficient mice develop less severe colitis when treated with acellular Bordetella pertussis vaccine containing filamentous haemagglutinin due to an increased IL-10 production in the intestinal mucosa and an increased apoptosis of Th1 cells \(^62\).
Conclusion

Although CD and UC are both chronic inflammatory diseases of the intestines their aetiology and pathogenesis seems to be different. In UC, it seems that an increased apoptosis of epithelial cells results in the destruction of the epithelial barrier, so that intestinal components, e.g. bacteria and food particles have excess to the LP where they induce an inflammatory reaction. On the contrary, CD is probably the cause of an intrinsic defect in the apoptotic pathway of (autoreactive) T cells. Therefore, new therapeutic approaches have to focus on the different aspects that are involved in the apoptotic pathways in UC and CD.
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