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Review

Histopathological Evaluation of Apoptosis in Cancer

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Background

The first observations of programmed cell death were made more than a century ago, and the term "apoptosis" was coined for this widely occurring phenomenon as early as 2 decades ago [1,2]. However, it is only during the past few years that serious mechanistic studies on it have been launched, leading to a revelation of its basic molecular intricacies. Along with this, investigators also working with clinical tumor material have obtained new analytical tools to study the role of apoptosis in cancer. Indeed, during the past years, there have been numerous papers in which the occurrence and extent of apoptosis and its association with the growth and progression of cancer have been studied in various types of neoplasms. This is not surprising, because, in essence, tumor growth is the net result of cell proliferation and cell loss. Rather, one may wonder why it is that, after so many years of meticulous use of not only mitotic count but also of more sophisticated proliferation markers as pointers of cell growth, it is only now that "apoptotic index" is becoming to be included among the parameters used to measure tumor growth.

In the following, we first give a brief overview of the molecular mechanisms of apoptosis. We then look at the role of apoptosis in cancer. Finally, we review studies done on the occurrence and extent of apoptosis in various types of tumors, and on the apoptotic index as a prognostic marker. We also reflect on, as extracted from the literature and based on our own experience, the currently used methods to determine the number of apoptotic cells in tumor samples and their limitations.

Introduction

Apoptosis is a complex, tightly regulated, and active cellular process whereby individual cells are triggered to undergo self-destruction in a manner that will neither injure neighboring cells nor elicit any inflammatory reaction.[3–6] Three phases can be discerned in apoptosis: initiation phase, effector phase, and degradation phase.[7] In the initiation phase, the cells receive a stimulus triggering the apoptotic process. In the effector phase, apoptotic machinery is activated, but the process is still reversible.[7] In the degradation phase, a point of no return is reached, beyond which the cell disintegrates.[7]

The duration of the process of apoptotic cell death depends on the stimulus and the cell type and is usually estimated to take from 12 to 24 hours.[8] Visible changes in cell morphology last for 2 to 3 hours and are associated with the degradation phase.[7,8] The characteristic oligonucleosomal DNA fragmentation, manifesting itself as a ladder pattern in gel electrophoresis, is also a late event.[7,8]

Molecular Mechanisms of Apoptosis

Caenorhabditis elegans and the CED Genes

Much of our knowledge about the molecular mechanisms and regulation of apoptosis comes from the studies on *Caenorhabditis elegans*.[9,10] During development, 131 of its 1090 cells are lost through apoptosis.[9,10] This process is mainly regulated by three genes, *CED3*, *CED4*, and *CED9*, of which *CED3* and *CED4* are positive regulators of apoptosis, whereas *CED9* is antiapoptotic.[9,10] Homologous genes are found in higher organisms. The apoptosis-inducing caspases and apaf-1 correspond to *CED3* and *CED4*, respectively, whereas the well-known antiapoptotic bcl-2 corresponds to CED9.[11–13]

Induction of Apoptosis

A large number of stimuli can induce apoptosis in a cell type-dependent manner.[6,14] General inducers that act on most types of cells include various chemotherapeutic agents, ultraviolet and γ-irradiation, heat, osmotic imbalance, high calcium, and nitrogen oxide.[6,14] A selective induction, on the other hand, is seen, eg, in thymocytes that undergo massive apoptosis when exposed to glucocorticoids.[15] Also, ablation of a supply of a trophic hormone or a growth factor leads to apoptosis of only those cells that harbor the corresponding receptor.[16,17]

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Depending on the triggering factor and the cell type, there are multiple signaling pathways that lead to activation of the apoptotic machinery. A few of them are briefly mentioned here. Apoptosis induced by cytotoxic lymphocytes is mediated either by a nonsecretory, ligand-induced, and receptor-mediated mechanism or by a secretory perforin and granzyme-mediated mechanism.[18] In cases of DNA damage, apoptosis is initiated via p53-dependent pathway leading to activation of mediators such as bax and KILLER/DR5.[19,20] Withdrawal of a trophic factor, such as nerve growth factor in the case of neural cells or serum in the case of fibroblasts, triggers an apoptotic pathway mediated by p38 mitogen-activated protein kinase.[21] Changes in the composition of membrane phospholipids may also initiate apoptosis.[22–25] Radiation, for instance, leads to an activation of sphingomyelinase resulting in the degradation of sphingomyelin to ceramide.[22,25] Ceramide-mediated apoptosis is launched also by several other factors, such as serum deprivation, interleukin 1, tumor necrosis factor α, 1,25-dihydroxyvitamin D_3, and nerve growth factor withdrawal.[23]

One of the best-known apoptotic pathways is the one emanating from APO-1/FAS/CD95 receptor.[7,18,26–28] It belongs to a family of tumor necrosis factor-related receptor proteins and serves as a receptor for APO-L, a ligand present on cytotoxic T cells.[18,28] Its engagement leads to an ushering of cells toward apoptotic death.[18,28] Downstream of APO-1 is FADD/MORT1, which binds to the cytoplasmic end of APO-1.[28] Together, these two proteins form a death-inducing signaling complex with FLICE, a member of a large family of caspase proteins.[28,29] It is caspases that during the past few years have been extensively studied for their role in apoptosis. Indeed, they have been identified as a common final pathway of the execution of apoptosis in highly divergent systems.[28]

Caspases

Caspases are cysteine proteases that cleave their target proteins at aspartic acid residues in a defined consensus sequence context.[30,31] Presently, at least 12 caspases are known.[30] They are expressed as precursors and are activated in a cascade-like cleavage parade. It involves cleaving the molecule to 10- and 20-kd subunits, which then heterodimerize and associate into tetramers that constitute the active enzyme.[31,32]

The noncaspase target proteins include, eg, proteins of the DNA repair system, cytoskeletal or structural proteins, and oncoproteins.[33] Among the DNA repair enzymes is poly(ADP-ribose)-polymerase, which, after DNA damage, catalyzes attachment of ADP-ribose to nuclear proteins, such as histones.[33,34] Cytoskeletal or structural substrates include nuclear lamins, fodrin, cytokeratin 18, actin, and catenin β.[33,35–37] Best-known oncoproteins degraded by caspases are the retinoblastoma protein (Rb) and mdm2.[38,39] Recently, caspases have also been shown to activate DNase leading to chromosomal breakage of DNA during apoptosis.[40]

The bcl-2 Family

The bcl-2 family is another group of closely related proteins that plays a major role in apoptosis. It includes death-promoting and death-inhibiting members.[5,6,11,41–43] In a sense, they can be considered to operate at checkpoints in which it is determined whether a cell is ushered toward survival or death. They act upstream of caspases.[44]

Apoptosis-inhibiting members of the bcl-2 family include bcl-2, bcl-xL, bcl-w, bfl-1, brag-1, mcl-1, and A1.[5,6,41–43] Apoptosis-promoting members are bax, bak, bcl-xS, bad, bid, bik, and Hrk.[5,6,41–43] Instrumental for their action is homo- and heterodimerization, which occurs through their conserved domains.[5,6,41–43,45–47] They regulate apoptosis in a rheostatic manner; in an excess of bax, for instance, bax homodimers predominate, which favors apoptosis.[5,6] Conversely, in an excess of bcl-2, bcl-2/bax heterodimers are formed, which leads to inhibition of apoptosis.[5,6] Competition between family members also has an effect. bcl-xL, for example, inhibits apoptosis by binding and sequestering bax.[6] By binding bcl-2 and bcl-xL, bad, on the other hand, releases bax, which leads to bax homodimerization and promotion of apoptosis.[6]

bcl-2 is the epitome of an antiapoptotic or survival gene. Attesting to its role in an apoptosis checkpoint, it counteracts apoptosis initiated by quite disparate signals, such as chemotherapeutic drugs, oxidative stress, viral infections, and p53.[6] In lymphoid cells, for instance, bcl-2 inhibits apoptosis induced by glucocorticoids and growth factor withdrawal.[6] Indeed, in many cases, actions of bcl-2 underlie the well-known survival functions of hormones and growth factors. Thus, for example, in breast epithelial cells, estrogen stimulation leads to upregulation of bcl-2 and resistance to apoptosis.[48] Upregulation of bcl-2 and bcl-xL is also effected by interleukins.[49,50]

Many members of the bcl-2 family, such as bcl-2, bcl-xL, and bax, are resident proteins of the mitochondrial membranes, endoplasmic reticulum, and nuclear envelope in which they are inserted via their carboxy-terminal ends.[11,43,51] In mitochondria, they form pores and act as ion channels.[43,52–54] This is probably the key to their function in apoptosis. Namely, induction of apoptosis is almost invariably accompanied by disruption of the mitochondrial transmembrane potential and release of caspase-activating substances, such as cytochrome c and apoptosis-inducing factor, from the mitochondria.[43,52–54] Consistent with their role as negative regulators of apoptosis, induction of expression of bcl-2 and bcl-xL effectively counteracts the flow of these molecules to cytosol, whereas bax promotes it.[55,56] bcl-2 and bcl-xL counteract induction of apoptosis also by binding to apaf-1, which prevents it from activating pro-caspase-3.[12,13] A very recent study has demonstrated that caspase-3 is able to cleave the loop domain of bcl-2 at Asp[34], and that the carboxyl-terminal cleavage product triggers and accelerates apoptosis.[57] It remains to be seen whether in tumors showing a positive association between bcl-2 expression and the extent of apoptosis,

bcl-2 is present in a cleaved, and thus, in fact, apoptosis-promoting form.

Ever since its discovery as an upregulated gene in t(14;18) translocation in follicular lymphoma, bcl-2 has been considered as an "oncogene."[58] Now it has been revealed that its apoptosis-promoting countervail bax is a *bona fide* tumor suppressor gene. This is implied by a recent study of Rampino et al,[59] who have shown that more than 50% of colon cancers exhibiting the microsatellite mutator phenotype contain disabling somatic mutations in the Bax gene.[59] None of the microsatellite mutator phenotype-negative tumors showed any mutations. Given the role of bax in apoptosis, this strongly supports the notion that paralysis of the death machinery is, by way of leaving genetically injured cells uneliminated, an important step in the progression of cancer.

Cancer Genes and Apoptosis

One facet of the intertwinement of apoptosis and cancer is the involvement of many oncogene and tumor suppressor gene products in the regulation and execution of apoptosis. Among them are p53, Rb, ras, raf, and myc. p53, because of its role in apoptosis, has earned the name "guardian of the genome."[60] It monitors the state of DNA, and, in case of DNA damage, stalls the cell cycle.[60–62] This takes place through the induction of CIP/WAF1/p21, a protein that prevents phosphorylation of cyclin-dependent kinases, the well-known positive regulators of the cell cycle.[63,64] In the absence of phosphorylated, active cyclin-dependent kinases, also another regulator of the cell cycle, Rb, remains inactive (unphoshorylated), and, hence, the cell cycle halts.[65] This then leads to activation of DNA repair machinery. If the DNA repair fails, p53 takes over again and triggers apoptosis in a process that involves upregulation of the apoptosis-inducing bax and down-regulation of the anti-apoptotic bcl-2.[19,60,66,67] p53 also upregulates KILLER/DR5, a novel 45-kd apoptosis-inducing member of the tumor necrosis factor receptor family.[20,68] Analogous to the APO-1/FAS/CD95 receptor system, its activation also leads to a FLICE-mediated caspase activation.[20,68]

Proto-oncogenes *myc* and *ras* are also part of the apoptotic machinery. The role of *myc* is capricious, because it depends critically on how the cell is "conditioned" by other factors. Thus, in the presence of growth factors, it induces proliferation, whereas in their absence, it acts apoptotic.[69]

Overexpression of *ras* may lead to increased or decreased apoptosis.[70–72] It is negatively regulated by bcl-2.[73] Phosphorylation of bcl-2, however, invalidates its capacity to protect cells from *ras*-induced apoptosis.[73]

Morphology of Apoptosis

Several light and electron microscopically detectable changes characterize apoptosis.[1,3,4] They include, most conspicuously, condensation of the chromatin to sharply delineated granular masses along the nuclear envelope, shrinking of the cells, convolution of the cellular and nuclear outlines, and fragmentation of the nucleus.[1,3,4] Finally, the cell disintegrates into membrane-bound apoptotic bodies that contain, eg, nuclear remains, and that are quickly removed by neighboring macrophages.[1,4] Throughout this process, the cell membrane and the membrane encasing the apoptotic fragments retain their integrity.[1,4] Also, the lysosomes remain intact and, hence, lysosomal enzymes are not released to the surrounding tissues.[1,4] Consequently, there is no associated inflammation in apoptosis.[1,4]

Apoptosis and Necrosis

Necrosis, in contrast to apoptosis, is considered to be a passive and a much more vaguely regulated event, the nature of which is dictated more by the type of the external injurious agent than by the internal workings of the cell.[4] The most obvious difference is that, whereas necrosis leads to a destruction of a large group of cells in the same area, in apoptosis, only scattered cells are involved.[4] The basic mechanistic difference is that in necrosis, because of membrane damage, there is swelling of the cytoplasm and bursting of the cell, which leads to a release of lysosomal enzymes and to inflammation.[4] In apoptosis, the outer cell membrane remains intact and the entire process is "contained" without any harm done to the neighboring cells.[4]

There are also differences in cellular morphology. Unlike in apoptosis, in necrosis, the chromatin is never marginated. Rather, it is unevenly distributed as clumps that are irregular and poorly defined.[4] Moreover, there is no nuclear fragmentation, cellular shrinking, or "body" formation in necrosis.[4]

Although quite distinct by appearance and considered antithetical, necrosis and apoptosis have been recently shown to be mechanistically related, eg, in the following ways.[8,52] First, *in vitro*, certain stimuli are apoptotic at low doses but bring about necrosis when present in high doses.[8] Second, many stimuli, such as heat shock, hypoxia, viruses, radiation, nitric oxide etc, can induce both apoptosis and necrosis.[8] Third, at a tissue level, areas of necrosis are surrounded by a zone of apoptotic cells, suggesting that they are associated phenomena.[74] Relatedness between apoptosis and necrosis is also seen at a biochemical level. Depletion of intracellular ATP in human T cells shifts cell death from apoptosis to necrosis.[75] Furthermore, caspases 8 and 10, which are located upstream in the signaling pathway, can also provoke necrosis.[8] This, on the other hand, can be inhibited by the anti-apoptotic bcl-2 protein, suggesting that at least part of the signaling machinery is shared.[8]

The dual nature of the death process has remained enigmatic. One way to settle this "apoptotic paradox" is to view the death process as a dichotomous event, the direction of which is highly context dependent.[52] Experimental evidence for this includes observations indicating that stimuli that under normal conditions lead to apoptosis may initiate necrosis under conditions of low intracel-

Table 1. The Extent of Apoptosis in Various Types of Tumors

Tumor type	Apoptotic index %	Method	References
Non-Hodgkin's lymphoma			
High grade	8.80, 1.44, 3.23	T	96,97,98
Low grade	2.40, 0.38, 0.71	T	96,97,98
Lung carcinoma			
NSCLC	0.37	T	137
	0.95, 1.00	M	135,162
SQCLC	2.30, 2.10	M	136,141
	1.20, 2.30	T	134,163
AC	1.10, 1.50	M	135,141
	1.20, 1.30	T	134,163
SCLC	0.10, 10.9, 2.65	T	140,163,164
	10.6	M	141
Colon carcinoma	3.50, 1.90, 5.10	T	127,165,166
	3.60, 4.70	M	141, 167
Endometrial carcinoma	0.89–1.29, 1.18–5.15	M	111,112
Prostate carcinoma	5.40	T	119
	0.87, 0.41, 0.80	M	121,141,168
Gastric carcinoma	1.12–1.26, 1.48–4.83	M	81,126
	2.80–5.10, 3.69–4.10	T	81,150
Breast carcinoma	1.20	M	141
	0.76	T	106
Thyroid carcinoma	0.20–1.40	T	122
Liver carcinoma	0.73	T	129
Pancreatic carcinoma	0.59	T	130
Bladder carcinoma	0.54–1.24	T	142
Salivary gland tumors			
Benign	0.01	T	94
Malignant	0.42	T	94

NSCLC, non-small-cell lung carcinoma; SQCLC, squamous cell lung carcinoma; AC, adenocarcinoma; SCLC, small cell lung carcinoma. T, TUNEL assessment; M, light microscopic assessment.

lular ATP.[8,75] Also, the availability of apoptotic proteases, eg, activated caspases, direct the cell death pathway such that sudden and extensive damage may exhaust the apoptotic machinery, causing it to yield to necrosis.[52]

Morphological Detection of Apoptosis

Detection of apoptotic cells in tissue sections in, eg, tumors is possible because of characteristic morphological features that are manifest even in routinely stained sections.[1,3,4] Recently, more refined techniques have also been developed for tissue studies, which are based on the detection of apoptosis-specific biochemical changes or expression of apoptosis-associated proteins directly on tissue sections.[76–78] Thus, for instance, the fragmentation of DNA into 180 to 200-bp fragments, the biochemical hallmark of apoptosis, is being used in morphological analysis of apoptosis.[4] Such techniques make use of radioactive or nonradioactive labeling of the free ends of the DNA, allowing accurate identification of single apoptotic cells.[76,78] This technique, called *in situ* 3′-end labeling method, can be divided in two variants. In the first, DNA polymerase or its Klenow fragment is used to incorporate labeled nucleotides into fragmented DNA by *in situ* nick translation.[79,80] In terminal deoxytransferase-mediated dUTP nick-end labeling (TUNEL), on the other hand, terminal transferase is used to add labeled nucleotides into the 3′-end of the DNA.[80] For the detection of the radioactive label, autoradiography is used, whereas with nonradioactive labeling, usually an appropriate chromogen reaction is used.

Apoptotic Index

In histological tumor material, apoptotic index is used as a measure of the extent of apoptosis. Most often it is defined as a percentage of apoptotic cells and bodies per all tumor cells. Some authors, however, use it to denote the number of apoptotic cells per 1000 tumor cells.[81] Furthermore, in some investigations, apoptosis is measured as number of apoptotic cells per 10 high-power fields.[82]

Table 1 shows a comprehensive list of studies with apoptotic indexes reported for different types of tumors by using either DNA end-labeling techniques (TUNEL) or plain morphology. In all listed cases, the apoptotic index is given as a percentage of apoptotic cells in tumor cell population.

It is readily apparent that there is a wide variation in the extent of apoptosis not only between different tumors but also within a tumor type (Table 1 and Figure 1). In high-grade non-Hodgkin's lymphomas, for instance, the average apoptotic index varies between 1.4 and 8.8% and in small cell lung carcinoma between 0.1 and 10.9%. Even though there may be biological variations within the tumor groups (in carcinomas the relative number of grade I, II, and III lesions may vary, and in lymphomas different

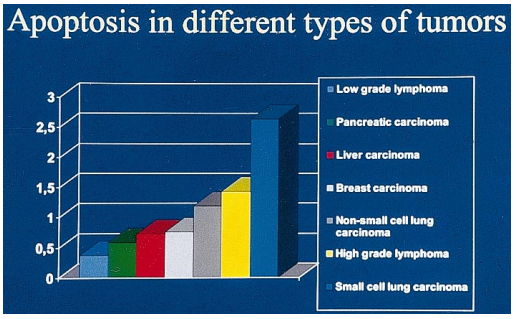

Figure 1. Variation in apoptotic index in different types of tumors in materials processed and analyzed in a similar manner (data are from Refs. 98, 106, 129, 130, 134, and 140). Of the tumors shown, low-grade malignant lymphomas have the lowest and small cell lung carcinomas the highest apoptotic index. The values presented represent averages, and wide variations between studies (as noted in Table 1) are common.

types of histological lesions may be variably represented), it is quite unlikely that it could solely account for the observed variability. Indeed, there are a number of methodological and technical factors that may influence the determination of the apoptotic index.

Detection of Apoptotic Cells

The extent of apoptosis may vary in different areas of the tumor and, frequently, apoptotic cells appear in clusters.[83] Consequently, to avoid erroneous results, care should be taken to include enough fields in the analysis. It is estimated that at least 20 microscopic fields of 1000× magnification (containing an average of 1500 cells each) should be examined to guarantee representativeness.[83]

Table 2. Factors Reported to Influence DNA End Labeling

Fixative used
Concentration of the fixative
Pretreatment used affects the results
Tissue drying
DNA strand breaks are not solely associated with apoptosis (fragmentation may be present in necrosis or may be caused by DNA-damaging agents)
Long fixation times (more than 3 weeks) may influence apoptosis
Delay in fixation
Duration and concentration of the polymerase or transferase enzyme treatment influence the number of nuclei stained

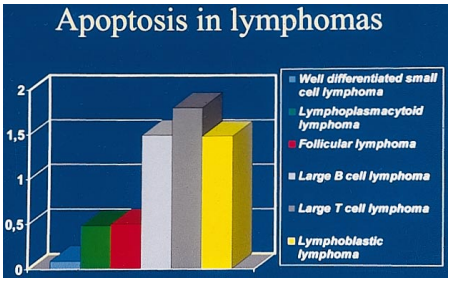

Figure 2. Difference in the extent of apoptosis between high-grade and low-grade lymphomas (data from Ref. 98). High-grade lymphomas display a significantly higher extent of apoptosis than do low-grade lymphomas.

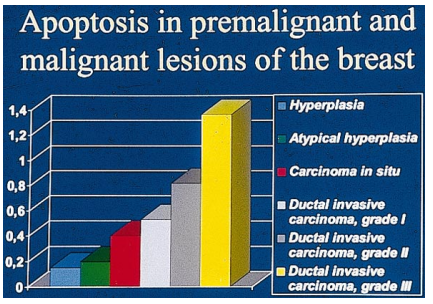

Figure 3. Relation of the extent of apoptosis with different types of breast lesions (data from Ref. 106). The extent of apoptosis increases parallel with the neoplastic potential of the breast lesion.

The identification of apoptotic cells also depends on the magnification used. With a lower magnification, fewer apoptotic cells are detected, and there is an increase in interobserver variability[83] Therefore, a high-power lens should be used.

One obvious cause for interobserver variation is error in counting the number of tumor cells in a given field. An erroneous estimate of the total number of tumor cells in the field easily changes the apoptotic index severalfold.

The duration of the morphologically detectable phase of apoptosis may vary and influence the apoptotic index.[84] In tumors in which detectable apoptotic changes take a longer time, the apoptotic index is higher, even though the actual number of cells undergoing apoptosis would be the same.[84] Because of this, the apoptotic

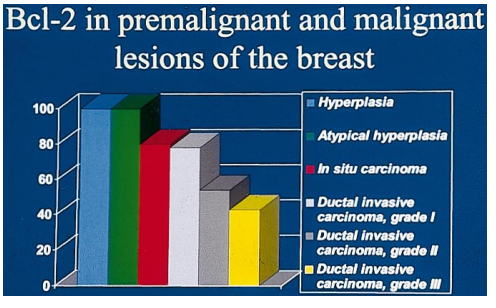

Figure 4. Relation of bcl-2 expression with different types of breast lesions (data from Ref. 106). bcl-2 is inversely related to the neoplastic potential of the breast lesion.

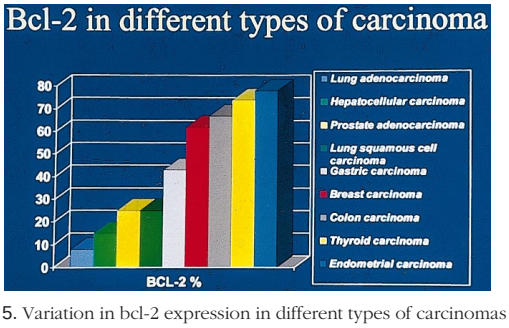

Figure 5. Variation in bcl-2 expression in different types of carcinomas (data are from references shown in Table 3). Highest bcl-2 expression is seen in thyroid and endometrial carcinomas, whereas lowest expression is seen in lung adenocarcinoma and hepatocellular carcinoma.

index cannot be correlated with an actual "death index," even though technical problems with measuring the apoptotic index could be solved.

Nonneoplastic apoptotic cells may also pose a problem for the estimation of the apoptotic index. This is the case especially in lymphomas, in which apoptotic macrophages and lymphocytes may resemble apoptotic neoplastic cells. Moreover, neoplastic cells may even stimulate apoptosis in reactive cells. This can take place if tumor cells express FAS-L on their surface.[85,86] The ligand may associate with the APO-1/FAS/CD95 receptor of the invading normal T lymphocytes and trigger apoptosis in them.[85,86] Apoptotic T lymphocytes mistakenly counted as apoptotic tumor cells would give a distorted view of the relationship between apoptosis and tumor growth.

False Positives and Negatives

Other sources of misinterpretation lie in the differences in the sensitivities and specificities of the end-labeling techniques and in such technicalities as, for instance, the type of pretreatment, the type of labeling enzyme, the method of tissue processing, and incubation times.[79,87,88]

It seems to be a common experience that performance of TUNEL labeling depends greatly on the tissue pretreatment, concentration of the terminal transferase enzyme, and the type and concentration of the fixative (Table 2).[80,87,89,90] Ethanol fixation, for example, is associated with diminished staining intensity and increased background staining compared with 10% buffered formalin.[88] Of the tissue pretreatments, microwave treatment of the formalin-fixed tissue raises the number of labeled cells twofold compared with proteinase K pretreatment.[80]

In the *in situ* 3′-end labeling method by *in situ* nick translation, a high concentration of or a prolonged incubation with polymerase leads to a gradual increase of the labeling of the morphologically normal nuclei.[79] The number and intensity of the labeled nuclei also depends on the duration of the tissue pretreatment with pepsin or proteinase K; a brief treatment leaves many apoptotic cells unlabeled, whereas too long a pretreatment results in labeling of morphologically nonapoptotic nuclei.[79,87] In the studies referred to in Table 1, proteinase K at a concentration of 20 to 40 μg/ml for 15 minutes was usually used. In many studies, however, there is no mention of the proteinase K concentration.

A further caveat of the end labeling methods is that cells other than apoptotic cells can also become labeled. Such false positives are seen especially in cases with DNA damage, autolysis, tissue drying, and necrosis.[87,89,91] Conversely, formalin fixation of a long duration can give rise to "false negatives" because of a decreased labeling of apoptotic cells.[92]

The lowest numbers of apoptotic cells are usually scored in light microscopy based solely on morphology (Table 1). This may be due to the fact that morphological manifestations of apoptosis, such as shrinking of the nucleus, are of such a short duration or inconspicuous that they could go partially undetected. Results obtained by "plain" morphology show, however, a good correlation with DNA end labeling methods.[93,94] Thus, morphology alone, although less sensitive, is a fairly reliable and inexpensive method for the detection of apoptosis.

Comparison of *in situ* nick translation and TUNEL shows that TUNEL is more sensitive.[87,88] This could be at least partially due to the ability of terminal transferase (used in TUNEL) to label both double- and single-stranded DNA breaks, whereas polymerase I (of *in situ* nick translation) only labels single-stranded breaks.[80,88] Also, the kinetics of the enzymes are different; DNA polymerase I is slower than terminal transferase in incorporating nucleotides.[87] In investigations on clinical tumor material, the TUNEL technique has almost exclusively been used.

Occurrence of Apoptosis in Human Tumors

Lymphomas

High-grade malignant non-Hodgkin's lymphomas show a significantly higher apoptotic index than low-grade lymphomas (Figure 2).[95–98] This correlates nicely with the occurrence of antiapoptotic bcl-2, which is overexpressed in low-grade follicular lymphomas due to a translocation that takes the bcl-2 locus to a highly active chromosomal environment.[58] In fact, there is an inverse association between the immunohistochemical expression of bcl-2 and the apoptotic index.[95,98] Also, other members of the bcl-2 group, such as the apoptosis-promoting bax and apoptosis-inhibiting mcl-1, are expressed in lymphomas.[98,99] They are also seen in Reed-Sternberg cells of Hodgkin's disease.[100,101] A potentially important factor is Epstein-Barr virus, which is found in Hodgkin's disease, in some other lymphomas, and also in some nonlymphoid neoplasms such as gastric and nasopharyngeal carcinomas.[102–104] Its latent membrane protein 1 upregulates bcl-2 and can, in this way, influence the extent of apoptosis.[105]

Breast Carcinomas

In breast carcinomas, a high extent of apoptosis is associated with a poor prognosis, and more apoptosis is seen in tumors of high grade (Figures 3 and 4).[82,106] This is probably due to a loss of receptors for hormones that act as survival factors. Interestingly, in breast carcinomas, concurrent expression of progesterone or estrogen receptors and of antiapoptotic bcl-2 can be seen.[82,107] This correlates with the cell culture studies that show that stimulation of the estrogen receptors leads to upregulation of bcl-2.[108] bcl-2 expression is seen in 70% of breast carcinomas, and its expression is inversely associated with the apoptotic index and with a better prognosis.[82,106,109,110]

Endometrial Carcinomas

Apoptosis is increased in high-grade endometrial adenocarcinomas and is more pronounced in tumor areas with a solid growth pattern.[111,112] To some extent, this corre-

Table 3. Immunohistochemical bcl-2 Expression in Carcinomas

Tumor type	bcl-2	Reference
Lung carcinoma		
SQCLC	35%	136
	28%	134
	36%	169
	25%	170
AC	0%	136
	8%	134
	12%	170
SCLC	93.70%	169
Colon carcinoma	67%	127
	5.3%	167
Endometrial carcinoma	50–78%	112
	47%	113
Prostate carcinoma	25%	120
Gastric carcinoma	43%	126
Breast carcinoma	62%	106
Thyroid carcinoma	74%	122
Liver carcinoma		
Hepatocellular	0%	133
	15%	132
Cholangiocarcinoma	60%	133
Pancreatic carcinoma	55%	171
Bladder carcinoma	24.7%	143

SQCLC, squamous cell lung carcinoma; AC, adenocarcinoma; SCLC, small cell lung carcinoma.

lates inversely with the expression of bcl-2, which is strongly expressed in epithelial cells of the normal endometrium but reduced in atypical hyperplasias and carcinomas.[113,114] The expression of bcl-2 is associated with a lower extent of apoptosis.[112]

Prostate Carcinoma

In prostate cancer as well, hormones acting as survival factors and their receptors play a role. It is well known that androgen deprivation leads to apoptosis of normal prostate epithelial cells and tumor cells.[115,116] Characteristic of prostatic carcinoma cells is that further down in the course of progression, they tend to gain resistance to the apoptosis-inducing hormone withdrawal.[117] High bcl-2 expression is found in androgen-independent prostate tumors, suggesting that bcl-2 upregulation contributes to the survival of neoplastic cells in a hormonally deprived environment.[118] In line with this, the extent of apoptosis was found to be lower in recurrent than primary tumors.[119] bcl-2 positivity is found in only 25% of prostate carcinomas and is reported to be higher in high-grade tumors (Table 3 and Figure 5).[120] Another antiapoptotic factor, mcl-1, is expressed in 81% of prostate carcinomas and is also more frequently seen in high-grade tumors.[120] Even though there is a decrease in apoptosis in recurring tumors, and the expression of antiapoptotic bcl-2 and mcl-1 is higher in high-grade tumors, increased apoptosis has been associated with a poor prognosis.[121]

Thyroid and Adrenal Tumors

More apoptosis is found in thyroid carcinomas with a low degree of differentiation.[122] There is an inverse association between the bcl-2 expression and apoptosis in papillary thyroid carcinomas but not thyroid cancers of other histological types.[122]

In adrenal cortical tumors, apoptosis is reported to be lower or at the same level as in nonneoplastic tissues.[123,124] In cell culture studies, bovine adrenocortical cells undergo apoptosis in adrenocorticotropic hormone-free culture conditions.[125]

Gastrointestinal Carcinomas

In gastric and colon carcinomas, lower apoptotic indexes have been reported in early than in advanced-stage lesions, but there are also opposing results.[81,126] Positive bcl-2 immunoreactivity is found in 43 and 67% of the gastric and colon carcinomas, respectively, and its occurrence seems to be inversely associated with apoptosis (Table 3 and Figure 5).[126,127] Stronger bcl-2 expression was associated with gastric carcinomas of the diffuse type.[128] On the other hand, expression of bax, mcl-1, and bcl-X was more frequent in the intestinal type.[128]

In pancreatic and hepatocellular carcinomas, no association is found between apoptosis and the grade of tumor or survival of the patients.[129,130] Among liver carcinomas, hepatocellular carcinomas show a much lower bcl-2 expression than cholangiocarcinomas (Table 2).[131–133] This is analogous to bcl-2 expression in the nonneoplastic liver; normal hepatocytes do not express bcl-2, whereas a high degree of expression is seen in small bile ducts.[131,133]

Lung Carcinomas

In non-small cell lung carcinomas (NSCLCs), no association between apoptosis and survival or advanced stage of the tumor is usually found, but again, there are also conflicting reports.[134–137] In NSCLCs, bcl-2 expression can be seen in only 8 to 30% of the cases, whereas in small cell lung carcinomas, it is present in 90%.[138,139] Curiously enough, small cell lung carcinomas have a higher apoptotic index than NSCLCs, even though their bcl-2 expression is high.[140,141]

Urogenital Carcinomas

In transitional cell carcinomas of the bladder, high-grade tumors display a higher apoptotic index.[142] bcl-2 expression is low, ranging from 5 to 25% in different reports.[142,143] A similar low frequency of bcl-2 expression is also found in transitional cell carcinomas of the renal pelvis and in renal cell adenocarcinoma.[144,145]

Other Tumors

Other tumor groups have been less extensively studied, and in many cases only single reports are available. In brain tumors, more apoptosis is seen in grade II gliomas compared with grade III lesions, suggesting that apopto-

sis contributes to a better prognosis.[146] Glioblastomas, however, have a higher apoptotic index than better-differentiated tumors.[146]

Also in germ cell tumors, apoptosis varies in histologically different types of tumors, being highest in the more aggressive and less-differentiated ones, such as embryonal carcinomas.[147] Interestingly, bcl-2 expression was only found in teratocarcinomas but not in other germ cell tumors.[147]

Apoptosis in sarcomas and mesenchymal tumors has not been studied extensively. The apoptotic index in most sarcomas is generally between 0 and 6%.[141]

Premalignant Lesions

In atypical hyperplasias and in *in situ* carcinomas of the breast, a rise in the apoptotic index and a decrease in the bcl-2 immunoreactivity is associated with the grade of the lesion.[106] In endometrial atypical hyperplasias and in esophageal Barrett's metaplasia or dysplasia, a decrease in bcl-2 immunoreactivity was also seen.[148] Thus, in these cases, the idea of less apoptosis promoting the early steps of carcinogenesis is not sustained, at least in its simplistic form. Rather, in light of these examples, a high degree of apoptosis in premalignant lesions can be considered to be a reflection of an ardent effort to eliminate genetically damaged cells. This, in fact, has been suggested in studies on apoptosis in gastric premalignant lesions and in dysplasias of the oral cavity, which show that the apoptotic index may be even higher in dysplastic lesions than in the corresponding invasive carcinomas.[149,150]

On the other hand, in dysplastic lesions of the colon and stomach, there is an increase of bcl-2 immunoreactivity compared with invasive tumors.[127,151,152] This finding has been appropriately interpreted to mean that dysplastic, most likely genetically compromised, cells thus become saved from apoptosis, which contributes to the neoplastic progression. Clearly, further studies are needed to get a comprehensive view on apoptosis and its regulating proteins in preneoplastic lesions.

Why Is There Increased Apoptosis in Cancer?

It is obvious from the considerations above that apoptosis is generally increased in cancer. In fact, there are only a few tumors, such as follicular B-cell lymphomas, in which inhibition of apoptosis has been convincingly shown to play a decisive role in the development of neoplasia.[58] On the other hand, occurrence of apoptosis does not show any single rule in its relation to tumor stage, grade, or progression. Rather, a high degree of tumor-dependent variability is seen.

Why then is it that apoptosis is so often increased in tumors? Part of the explanation probably lies in the activation of cancer genes in the process of neoplastic development, some of which also influence apoptosis. In such cases, the degree of apoptosis is a reflection of the internal functioning of the death machinery. In other cases, apoptosis is due to extrinsic factors such as activated T cells that launch a FAS-mediated apoptosis in tumor cells.

Table 4. Apoptotic Index and Proliferation

Tumor type	Method	Reference
Apoptotic index associated with proliferation		
Non-Hodgkin's lymphoma	Histone mRNA *in situ* labeling	96
	MIB1	97
NSCLC	Ki-67	136
	Mitotic count	135
Colon carcinoma	Ki-67	165
	Mitotic count	167
Gastric carcinoma	Mitotic count	126
Endometrial carcinoma	Mitotic count	112
Breast carcinoma	Mitotic count	82
Bladder carcinoma	Ki-67	142
Apoptotic index not associated with proliferation		
Gastric carcinoma	MIB1	81
Small cell carcinoma	PCNA	140
Pancreatic carcinoma	PCNA	130
NSCLC	PCNA	134

PCNA, proliferating cell nuclear antigen.

Loss of Cell Adhesion and Hypoxia Enhance Apoptosis

One cell biological explanation for apoptosis in tumor cells is the increased sensitivity to apoptosis of cells that have lost their matrix attachment or cell-cell contacts.[153] This could be due, for instance, to a loss of an expression of cell adhesion molecules from the surface of the neoplastic cells. Especially important in this sense are integrin and cadherin molecules.[154] Still another factor that is conducive to apoptosis in tumors are the hypoxic conditions that prevail in tumors. For instance, in experimental brain necrosis of rats, apoptosis is seen primarily in the area bordering the ischemic zone.[74] Also in tumors, an increased number of apoptotic cells are seen adjacent to necrotic areas.[155]

Apoptosis and Proliferation Are Mechanistically Linked

A consistent feature in many studies is the positive correlation or association between apoptosis and proliferation, suggesting that they are mechanistically linked (see Table 4). One link relates to the fact that although apoptosis may be initiated in any phase of the cell cycle, the majority of cells undergo apoptosis primarily in the G1 phase of cycling cells.[156] In fact, many proteins that operate in the cell cycle checkpoints are also regulators and inducers of apoptosis. Examples of such are p53 and Rb proteins, which act on the G1/S checkpoint. Also, overexpression of cyclins, such as cyclins D1, A, and B, can induce apoptosis.[157–161]

Concluding Remarks

Even though there is a high degree of variability in the apoptotic index reported by different authors for the

Table 5. Apoptotic Index and Survival

Tumor type	References
Prognosis not associated with a high apoptotic index	
NSCLC	135, 137
Small cell carcinoma	140
Gastric carcinoma	126
Thyroid carcinoma	122
Hepatocellular carcinoma	129
Poor prognosis associated with a high apoptotic index	
NSCLC	134
Breast carcinoma	82
Poor prognosis associated with a low apoptotic index	
Colon carcinoma	167

same types of tumors, some generalizations on apoptosis and its associations with some clinical and biological parameters can be made. In lymphomas and hormone-dependent epithelial tumors, such as breast, endometrial, or thyroid carcinomas, a higher extent of apoptosis is associated with tumors of a higher grade. This is in contrast to other epithelial tumors, in which association with tumor grade is variable and less evident. Another feature is that the apoptotic index in most tumors is associated with cell proliferation, suggesting a mechanistic link between these two mechanisms.

Because proliferation and apoptosis contribute to tumor growth, some authors have created compound indexes taking into consideration the influence of both proliferation and apoptosis and even necrosis.[129,143,150] In these studies, the ratio between apoptotic and mitotic index was higher in dysplasias than in invasive carcinomas, suggesting that apoptosis is overwhelmed by cell proliferation in invasive lesions.[145,149] In hepatocellular carcinomas, tumors showing a high extent of proliferation and a low extent of apoptosis and necrosis had a significantly worse prognosis than other tumors.[129] Thus, it seems to be more reasonable to combine apoptosis and proliferation, and perhaps also include necrosis, to a common index when evaluating their impact on tumor growth and prognosis in various neoplasias. Indeed, when evaluated alone, the extent of apoptosis does not generally associate with survival (see Table 5).

Finally, the studies show that in the evaluation of the apoptotic index there are technical and methodological problems. To reduce interobserver variations, a consent on the criteria of how to define and calculate the "apoptotic index" is needed. As far as the methodological factors are concerned, at least some standard protocols on tissue processing, protease pretreatments, incubations, etc., should be pursued. Also, applying more than one method to determine apoptosis would improve the accuracy and reliability. For instance, inclusion of a plain morphological evaluation of apoptosis with the 3′-end labeling method would be an inexpensive and straightforward complement to the investigation.

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