News and Commentary

Cancer, apoptosis, and nonimmune surveillance

G Klein*

Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden
* Corresponding author: G Klein; E-mail: Georg.Klein@mtc.ki.se

Programmed cell death is vitally important, in the strict sense of the word, for life. The work of Bob Horvitz has been pivotal in understanding the molecular mechanisms of apoptosis in C. elegans. This study has also shown the role of apoptosis in development and the repair of DNA damage.

Apoptosis is one of the main mechanisms that protect us against oncogene-driven illegitimate growth with a malignant potential. Were it not for this and other nonimmune surveillance mechanisms, we would all die of cancer before we reach puberty. It is fair to say that no malignancy can develop unless the apoptotic machinery is severely impaired by additional genetic and epigenetic changes. The basic features of this machinery, its regulation, its crosstalks as well as the most recent therapeutic approaches aiming at its restoration are all based on Bob Horvitz' and his colleagues' discovery of the basic and highly conserved mechanisms that are fully developed in the little worm.

The need for protection against the danger of dysregulated growth from within is as mandatory for multicellular organisms as the protection against outside invaders. Surveillance against malignancy has been first mainly thought of in immunological terms (for a review, see Klein[1]). Later, it turned out, however, that immune surveillance constitutes only a very small part of the numerous mechanisms that protect us against cancer[2]. It acts mainly against virally transformed cells that provide virally encoded, immunogenic protein targets. These proteins are indispensable for the induction and maintenance of the transformed state, and can therefore serve as stable targets. Nonviral tumors are sculpted by multiple genetic and epigenetic changes that also include escapes from immune recognition.

What are, then, *the non-immune surveillance mechanisms* that keep ever-threatening cancer cells at bay?

They can be considered under the following four categories: *genetic* (mainly DNA repair), *epigenetic* (chromatin structure), *intracellular* (apoptosis, growth arrest), and *intercellular* (contact inhibition, matrix dependence).

These categories are closely interdependent. Apoptosis can be seen as a common effector between at least three of them.

Genetic (DNA repair-based) surveillance

The best-known form acts through the p53-dependent pathway. Upon DNA damage, wild-type p53 protein is stabilized, its level rises, it binds to DNA and induces growth arrest. During the ensuing interval, DNA repair can take place. In cells that successfully repair their DNA, p53 levels decrease and cell division starts again. Cells with persisting major DNA damage eliminate themselves by apoptosis.

More than 50% of all human tumors carry p53 mutations. They impair the DNA-binding capacity of the protein. Cells with damaged DNA but with maintained mitotic capacity go on dividing. They provide a great variety of mutants that can serve as the basis for the Darwinian play of the malignant microevolution.

Tumor risk is highly influenced by mutations in genes that control the fidelity of DNA replication, the efficacy of DNA repair, and chromosome separation checkpoint controls. Mutations in these genes, whether registered as point mutations, microsatellite instability, or loss of heterozygosis, are referred to as *mutator mutations*. The highly relevant DNA damage response pathways in the nematode *C. elegans* are summarized in this volume by Stergius and Hengartner[3].

Xeroderma pigmentosum (XP) is the oldest known case of a specific DNA repair deficiency. It is due to recessive mutation in one of the essential components of the nucleotide excision repair (NER) system, the repairosome (composed of 30 different proteins), that excises thymidine dimers from the DNA of UV-exposed skin.

XP patients must protect their skin from light all their lives, but they nevertheless develop multiple skin carcinomas. This emphasizes the paramount importance of DNA repair as a first-line surveillance mechanism.

Hereditary non-polyposis colon cancer (HNPCC) is due to a defect in one of several DNA mismatch repair (MMR) genes. Their protein products are involved in splicing out the mismatched region and inserting new bases to fill the gap.

MMR defects can be manifested as microsatellite instability (MSI) that is associated with multiple cancers. MLH1 is one of the frequently involved genes, both in the hereditary and the sporadic cases. MLH1 mutation in the hereditary cases and epigenetic silencing by dense hypermethylation of the 5' promoter region give the same MSI phenotype.

Epigenetic surveillance

This field is in its incipient phase. Its existence is suggested by the recent work of Ciu et al[4], showing loss of imprinting (LOI) of the IGF2 gene in the normal colonic mucosa of 30% of colon cancer patients, but in only 10% of healthy individuals. The authors suggested that LOI may serve as a predictive marker of an individual's risk for colorectal carcinoma. Experimental studies showing inherited interindividual variation in the frequency of hypermethylated CpG islands of tumor-suppressor genes and other epigenetic traits[5] also suggest the existence of epigenetic surveillance.

At the somatic level, the possibility to induce differentiation in certain leukemias and, more rarely, solid tumors, by natural

maturation-inducing factors or by chemicals such as phorbol esters or DMSO, demonstrate the lability of epigenetic states in some tumors that can be tipped from proliferation to differentiation[6].

Proliferation or differentiation are not the only alternative choices that may be made by tumor cells. Differentation or apoptosis is another. This can be illustrated by a recent experiment of Jain *et al.*[7] Osteosarcomas and T-cell lymphomas were induced with a tet-suppressible v-myc construct. In the absence of tet, the tumors grew progressively *in vitro* and *in vivo*. Temporary suppression of v-myc expression by tet has led to terminal differentiation with bone production in the sarcomas and to apoptosis in the T-cell lymphomas. More than two decades earlier, Holtzer, Böttiger, Graf, and Beug showed in their respective systems that temporary downregulation of a temperature-sensitive v-src that was used to transform melanoblasts, chondroblasts, osteoblasts, and fibroblasts, and of temperature-sensitive v-erbB that was used to transform chicken erythroblasts, permitted the cells to go on to terminal differentiation to their respective phenotypes, and they could no longer grow as tumors.[8,9]

These experiments suggest that oncogenes like src, erbB, and myc block the progression of differentiation in a variety of target cells and thereby prevent the exit of the cells from the cycling compartment. Release of the block by transferring the cells to the nonpermissive temperature allows them to proceed to terminal differentiation. T-lymphocytes do not have this option, however. In the absence of specific survival or growth factors, they trigger the death program.

At a more general level, the following scenario may be suggested:

Illegitimately activated oncogenes drive the proliferation of malignant cells. This can only occur in cells where one or several major apoptotic pathways have been inactivated. If oncogene expression is inhibited, the cell may activate its terminal differentiation program, without triggering its residual apoptotic pathways. If that option is not available, an apoptotic pathway is activated. This implies that apoptosis inhibition in tumor cells is always relative, rather than absolute. In fact, radiotherapy and chemotherapy are believed to act, wholly or partially, by inducing apoptosis in the tumor target.

Intracellular surveillance

The cell has multiple safeguards of great evolutionary depth that can prevent the progressive growth of illegitimately activated cells. The need for such controls must have arisen already in the earliest metazoa.

Apoptosis and growth arrest

Apoptosis and growth arrest are the most prominent intracellular controls in cells driven by illegitimately activated oncogenes. Apoptosis is steered by multipathway, multistep programs that terminate in the enzymatic breakdown of cellular DNA. It can be initiated either through the extrinsic death receptor ligand or the intrinsic mitochondrial pathway.[10] Most programs converge towards the activation of caspases that cleave cellular substrates, leading to characteristic biochemical and morphological changes. There are also caspase-independent pathways of cell death, however.

Many facts have been gathered on the major cellular apoptotic signaling cascades. The mitochondrial pathway is associated with activation of the APAF-1- and caspase-9-containing apoptosome. The death receptor pathway acts through caspase-8. The best-known p53-mediated apoptosis contributes mainly to the activation of the former, and exerts its effect through transcriptional regulation of its target genes. The mitochondrial pathway is regulated by the bcl-2 protein family, which includes both proapoptotic and prosurvival members. The proapoptotic proteins include the 'BH3-only domain' mediators of apoptosis. In response to DNA damage, at least two of them, Puma and Noxa, are transcriptionally induced, in a p53-dependent manner. Post-translational phosphorylation of BAD and BLK and proteolytic cleavage of BID are additional mechanisms for the regulation of the 'BH3 only domain' proteins in response to an apoptotic stimulus. The signal is then relayed to the multidomain proapoptotic proteins BAX and BAK, initially kept in an inactive state. Upon activation, conformational changes promote their stable association with the mitochondrial membrane, either to form a pool or to interact with channel-forming proteins and increase membrane permeability. Cytochrome *c* and other intermembrane space proteins are then released to efficiently induce caspase-dependent and/or independent apoptosis.

Many physiological growth control mechanisms that govern cell proliferation and tissue homeostasis are linked to apoptosis. It is therefore logical that a relative resistance of tumor cells to apoptosis is an essential feature of cancer cell development.[11]

Cell death machinery can be disabled in cancer cells at many different levels

They include the overexpression of apoptosis inhibitors (e.g. BCL2), inactivating mutations of executioner caspases, downregulation and mutation of proapoptotic genes (like BAX, APAF1, CD 95), alterations of the PI3K/AKT pathway and others.

Similarly to other tumor-related genetic changes, like oncogene activation and tumor-suppressor inactivation, many different genetic and epigenetic changes can bring about the same or similar phenotypic effects. Moreover, each pathway can be affected at different alternative levels. This will be illustrated by some examples.

Tumor cell escape from apoptosis

Tumor cell escape from apoptosis through the elevated expression of antiapoptotic molecules may affect the proximal level of apoptotic threshold. For example, bcl-2 can be upregulated by a chromosomal translocation to an Ig locus. It can also occur at the distal level of the caspases. The inhibitor of apoptosis (IAP) protein family is a case in point, with survivin as an outstanding example.[12] The IAP family has presently eight members in humans. They all have a similar structure and inhibit caspases. The pattern of IAP expression

is reminiscent of the cancer testis (CT) antigen group, known from cancer immunology, expressed in testis and in many different cancers, but not in adult tissues.

Survivin has a highly tumor-specific expression in adults. Loss of p53 and overexpression of survivin is a powerful apoptosis suppressing combination, more effective than either alone. A mutant of survivin, T34A, antagonizes survivin expression, induces apoptosis, and suppresses tumor growth *in vivo*. It does not damage normal cells.

The exogenous apoptotic pathway, based on the triggering of the death receptor Fas by Fas ligand, may also be impaired in the course of tumor development and progression. Loss of Fas and gain of aberrant FasL expression are common features of the malignant change. Loss of Fas expression is a prerequisite of aberrant FasL expression, which would otherwise induce suicide or fratricide among the tumor cells. Other malignancy-related changes like ras or p53 mutations downregulate Fas expression. Germline mutations of Fas in humans are associated with a high risk of lymphoid and solid tumors.

Activation of AKT is also an important survival mechanism of tumor cells. AKT phosphorylates and inactivates BAD and procaspase 9. It can be triggered by upstream signaling molecules, such as ras or receptor tyrosine kinases. It activates IkB kinase, a positive regulator of NF-κB, leading to the transcription of antiapoptotic genes. Activated AKT prevents the release of cytochrome *c* and antagonizes proapoptotic factors such as Fas ligand. Tumors with an upregulated AKT pathway become independent of survival signals.

Among the mutated and downregulated proapoptotic genes, apoptotic protease-activating factor (APAF-1) plays a prominent role. It interacts with cytochrome *c* and caspase 9 in the 'apoptosome' to mediate p53-dependent apoptosis. Melanomas show a high rate of LOH in the APAF-1 gene (more than 40%). The second allele is usually inactivated in metastatic (but rarely in primary) melanomas. It can be reactivated by 5-aza C.

Oncogenes and apoptosis

Proapoptotic oncogenes include myc, ras, E2F1, and E1A. They upregulate APAF-1 and procaspase 9. A rise in the APAF-1 level increases the sensitivity of apoptosome activation and cytochrome *c* release. Oncoproteins activate the apoptosome at the point of holocytochrome *c* release from mitochondria due to mitochondrial outer membrane permeabilization. This is a sudden process that involves all mitochondria.

Integrin receptors

Proper attachment to the extracellular matrix, and especially to basal membrane, is mediated by integrin receptors. It allows the cells to gain proper polarity, assemble their cytoskeleton, and resist proapoptotic stimuli, like those mediated by tumor necrosis factor related apoptosis inducing ligand (TRAIL), Fas, and TNFα. The antiapoptotic state is correlated with cellular resistance to chemotherapeutic agents.

Integrin signaling molecules that promote cell survival are focal adhesion kinase (FAK), Shc, and ILK. Each of them may impinge on the AKT/P13 K pathway. Inactivation of the tumor-suppressor gene PTEN constitutively activates both ILK and AKT.

Death by neglect

Normal cells require survival signals. Lack of such signals triggers apoptosis. Survival signals include growth factors, cytokines, hormones and other stimuli. Some of them are mediated by adhesion molecules. They are transduced by the PI3K/AKT pathway.

Anoikis is a special case of 'death by neglect'. It is triggered by inadequate or inappropriate cell matrix contacts. Anoikis maintains the correct cell number in epithelial tissues. The breakdown of anoikis contributes to neoplasia. It conveys selective advantage on precancerous epithelial cells. Resistance to anoikis may facilitate metastasis by allowing cells to survive following detachment from the matrix.

Is inactivation of the Rb and p53 pathways a universal rule in neoplasia?

It appears so. Tumors that carry wild-type p53 have, as a rule, inactivated the pathway at other levels, for example, by ARF mutation, upregulation of an ubiquitin ligase, MDM2, that interacts with ARF and prevents it from binding to p53 and targeting it for destruction in the proteasome. The Rb pathway is inactivated by mutation or loss of Rb itself, by p16 inactivation (often by methylation), or by CDK4 amplification.

Interestingly, both the Rb and p53 pathways are targeted by the small- or medium-sized DNA tumor viruses, such as SV40, the oncogenic adenoviruses, and papillomaviruses. The same two pathways are inactivated in most and perhaps all tumors of nonviral origin. This is the clearest common denominator between tumors of viral and nonviral origin.

Attempts to decrease the apoptotic threshold of cancer cells

All cancer cells have an elevated apoptotic threshold. Relative resistance against apoptosis is an equally essential part of the neoplastic evolution as the dysregulation of the cell cycle. No tumor cells are completely resistant to apoptosis, however.

The therapeutic effect of irradiation and chemotherapy has been previously attributed to their direct genotoxic effect on the tumor cells. While these agents can affect DNA *in vitro*, they can only do so at doses that exceed the therapeutic doses by several orders of magnitude. It is now clear that the therapeutic effects are due to apoptosis induction. Development of resistance against genotoxic treatment may be associated with further increase of the apoptotic threshold.

Is it possible to reduce the apoptotic threshold in cancer cells?

Exploitation of the TRAIL-stimulated apoptotic pathway appeared as an interesting possibility.[13] TRAIL-mediated apoptosis is independent of p53 status and of bcl-2. Normal cells are resistant, cancer cells are relatively sensitive to

TRAIL-stimulated apoptosis. *In vitro*, 80% of human cancer cell lines, derived from colon, lung, breast, skin, kidney, and brain tumors were sensitive to TRAIL, whereas most normal cell types were resistant. The reasons are not clear, but the findings raised hopes that recombinant TRAIL would have a therapeutic potential. Treatment of chemoresistant cancer with TRAIL was a particularly interesting possibility. This approach suffered a (hopefully temporary) setback, however, due to strong apoptosis induction in normal hepatocytes.

Apoptosis induction in cancer cells may also be achieved by what may be referred to as the 'Achilles heel approach'. Tumor cells driven by highly active proliferation-stimulating genes, triggered gene by amplification or by gene fusion, may be dependent on the elevated activity. Two partially effective agents that target such highly expressed oncogene products, Herceptin and Gleevac, may illustrate the point. Herceptin, a humanized antibody, is directed against the product of the erb B2 oncogene that is amplified in about 25% of breast carcinomas. The antibody has a favorable effect on tumors with this amplification.

Gene amplification has been extensively studied as a mechanism of drug resistance. For instance, treatment of bacteria or leukemic cells with methotrexate leads to the development of resistance accompanied by the amplification of the dihydrofolate reductase (DHFR) gene. But amplification is only maintained under selection pressure. It has been argued that the maintenance of amplified oncogenes in tumor cells indicates that their high expression level has become essential for the survival and/or proliferation of the cells.

Herceptin is not a cytotoxic antibody. There is some evidence that it acts by inducing a downmodulation of erbB2 expression. The cells die by apoptosis.

A similar mechanism may explain the effect of Gleevac, an inhibitor of the high tyrosine kinase activity of the bcr-abl fusion protein that can bring Ph1, positive CML, and ALL cells to regression.[14]

Many similar cases of specific 'Achilles heel targeting' will be undoubtedly developed in the future. The risk of resistance development is high, as already demonstrated both for Herceptin and Gleevac. Like in cytotoxic chemotherapy several decades earlier, resistance will have to be dealt with combining several independently acting targeting agents.

Many other apoptosis-based clinical trials are now in progress. Attempts include specific inhibitors of proapoptotic proteins, such as bcl-2, angiogenesis inhibitors, modified adenoviruses (Onyx) that may specifically kill p53-deficient cells. The experience with Onyx was particularly instructive. At a time when it was known that 50–60% of all human tumors carry mutated p53, an E1A-defective adenovirus was built that could lytically multiply in cells with mutant but not with wild-type p53. E1A normally inhibits p53, as an important part of the viral strategy. It turned out, however, that the Onyx virus would multiply in tumor cells no matter, whether they carried mutant or wild-type p53. In view of the fact that the p53 pathway is impaired in virtually all tumors, either at the level of p53 itself, or at another point in the pathway, this is fully understandable.

Other approaches involve the use of small molecular inhibitors of histone deacetylase and other proteins that are involved in pathways tumor cells use to escape apoptosis.

Another intensively pursued approach is the attempt to activate mutant p53 by gene transfer or by molecules that reestablish wild-type configuration in mutant p53. Inhibition of the negative p53 regulator MDM2 in tumors that carry wild-type p53 is another possibility. Targeting of the EGF receptor and antisense inhibition of bcl-2, particularly in lymphomas and myelomas, also aim at the rehabilitation of apoptosis in cancer cells with specific defects. It is likely that the best results will be achieved by treatments that focus individually on reconstructing the pathway that has been damaged in the targeted cancer cell itself.

Intercellular surveillance

This is a relatively unexplored area that may or may not be related to the field of integrin receptors.

In the 1960s, Michael Stoker, Leo Sachs, and Georges Barski have separately shown that normal cells can inhibit the growth of cells transformed by polyoma, chemical carcinogens or X-irradiation, when they are plated together in mixed culture. Cells transformed by the same virus did not contact inhibit each other, but cells transformed by different viruses, such as polyoma and SV40, did. Michael Stoker speculated that these observations may explain the phenomenon of tumor cell dormancy and the inability of many circulating tumor cells to give rise to metastasis.

The more recent observation is that extracellular matrix, including basal membrane attachment, is essential for epithelial cells to survive. Detachment leads to apoptosis, as already discussed. Neoplastic cells protect themselves by the switch on of AKT or survivin.

Numerous suppressor genes that are lost or inactivated during tumor evolution are normally involved with the maintenance of tissue architecture. Examples include cadherin, APC, and DCC.

Conclusions

All organisms have powerful surveillance mechanisms that prevent the outgrowth of potentially cancerous cells. Many of them are highly conserved by evolution. To this end, the *C. elegans* animal model has been essential.

Believed for a long time as the most important safeguard, *immunological surveillance* now occupies a relatively minor place. It is still a powerful restraint against the outgrowth of virally transformed cells, however. This is the reason why tumorigenesis by Epstein–Barr virus, the papillomaviruses, and HHV-8, the Kaposi sarcoma herpes virus, is either restricted to or more pronounced in the immunodefectives.

Non-immune surveillance is of four different kinds:

(i) *Genetic surveillance* is largely based on DNA repair. It is the first line of defense, robustly built on a multitude of repair mechanisms. Defects in repair enzymes lead to specific cancer syndromes, several of them associated with multi-cancer families.

(ii) The evidence for *epigenetic surveillance* is not yet firmly established. Preliminary evidence indicates the existence of inherited differences in the stringency of imprinting, possibly related to cancer risk.

(iii) *Intracellular surveillance* prevents the outgrowth of cells driven by illegitimately activated oncogenes. Growth arrest and apoptosis are its two main arms. They are related but distinct. Growth arrest can be assigned to specific tumor-suppressor genes, some of which are also linked to apoptotic pathways. Apoptosis is a firmly built multipathway system, as tightly controlled as cell division. Tumor development includes impairment or damage to one or several apoptotic pathways. Tumor cells use multiple escapes from apoptosis, including both debilitation of proapoptotic pathways (e.g. p53) and activation of antiapoptotic mechanisms, for example, AKT/PI3 K. Nevertheless, no tumor cell is completely resistant to apoptosis. 'Genotoxic' agents such as irradiation or chemotherapy act by inducing apoptosis in relatively apoptosis-resistant cells.

Many therapeutic approaches, including clinical trials, aim at the reactivation of apoptosis. Partial or full restoration of a pathway that has been damaged in a given tumor cell is most likely to succeed. The 'Achilles heel' approach involves mRNA or protein targeting, to reduce abnormally high enzyme levels and/or amplified oncoproteins.

(iv) *Intercellular surveillance* is less well explored. It is clear, however, that loss of contact by epithelial cells leads to anoikis, a special form of apoptosis. Inhibition of tumor growth by adjacent normal cells is another phenomenon of great interest, largely unexplored by molecular methods. It may explain long-range dormancy and counteract the outgrowth of disseminated tumor cells.

1. Klein G (1975) Harvey Lectures 69: 253
2. Klein G and Klein E (1977) Proc. Natl. Acad. Sci. USA 74: 2121–2126
3. Stergius L and Hengartner M (2004) Cell Death Differ. 11: 21–28
4. Cui H *et al.* (2003) Science 299: 1753
5. Paz MF *et al.* (2002) Cancer Res. 62: 4519–4524
6. Klein G (2002) Semin. Cancer Biol. 12: 327–330
7. Jain M *et al.* (2002) Science 297: 102–104
8. Boettiger D *et al.* (1977) Cell 11: 881–890
9. Weintraub H *et al.* (1982) Cell 28: 931–940
10. Green DR and Evans GI (2002) Cancer Cell 1: 19–30
11. Igney FH and Krammer PH (2002) Nat. Rev. Cancer 2: 277
12. Reed JC (2001) Clin. Investig. 108: 965–979
13. Nicholson DW (2000) Nature 407: 810–816
14. Gambacorti-Passerini C *et al.* (1997) Mol. Dis. 23: 380–394