Programmed Cell Death (Apoptosis) and Cancer Chemotherapy

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**Introduction**

The growth of any tissue, whether normal or malignant, is determined by the quantitative relationship between the rate of cell proliferation and the rate of cell death. In the adult, these cell kinetic rates are balanced such that neither involution nor overgrowth of tissues normally occurs. Because a cell must undergo a series of molecular changes to acquire the malignant phenotype and because these changes are often induced by agents or treatments that damage the cell over an extended period of time, any events that derange this kinetic balance in favor of an enhanced survival of initiated or damaged cells promote the carcinogenic process. In this regard, certain promoting agents of carcinogenesis function not to enhance proliferation but rather to decrease the death of neoplastic-initiated cells.[1] Thus, understanding what normally regulates cell death and how this regulation is deranged in carcinogenesis has important ramifications for chemoprevention. Also important for therapy of established cancers is the realization that a fundamental requirement for full transformation of premalignant cells into cancer cells is that they must undergo a series of genetic changes that disrupts this homeostatic balance. This disruption allows the rate of proliferation of cancer cells to exceed cell death, resulting in the net continuous accumulation of malignant cells. It is this continuous net accumulation of malignant cells that produces the lethality of cancer.

A successful therapy for established cancers requires readjusting the quantitative cell kinetic relationship so that cell death exceeds cancer cell proliferation. If this negative relationship can be maintained, eradication of the cancer is possible and the patient can be cured. However, no therapeutic regimens are presently available to accomplish this goal repeatedly when used against solid cancers such as breast, prostate, lung, and colon. An understanding of what normally regulates the balance between cell proliferation and cell death in these tissues is critical to maintain this negative relationship.

**Programmed Cell Death (Apoptosis)**

Unlike the detailed framework that is rapidly becoming defined in both molecular and cell biological terms for cell proliferation, an understanding of the initiation of cell death and the cellular mechanics of this process are just beginning. Cell death can involve processes that are equal in complexity and regulation to those involved in cell proliferation. This knowledge has been appreciated for many years by developmental biologists, who have coined the term “programmed cell death” (PCD) to distinguish the active, orderly, and cell-type-specific death observed in developing organisms from necrotic cell death. Necrotic death is a response to pathologic changes initiated outside of the cell and can be elicited by any of a large series of factors that result in a change in the plasma membrane permeability. This increased permeability results in cellular edema and in the eventual osmotic lysis of the cell. In necrotic cell death, the cell has a passive role in initiating the process of death. In PCD, a cell undergoes an energy-dependent process of cellular death initiated by specific signals in an otherwise normal microenvironment. In PCD, the cell is an active participant in its own demise.[2,3]

PCD is a widespread phenomenon that occurs normally at different stages of morphogenesis, in the growth and development of metazoans, and in normal turnover in adult tissue.[4] Under these physiologic conditions, PCD is initiated in specific cell types by both endogenous tissue-specific agents (generally hormones or locally diffusing chemicals) and exogenous, cell-damaging treatments (eg, radiation, chemicals, and viruses). Endogenous activation of PCD occurs either due to the positive presence of a tissue-specific inducer (eg, glucocorticoids that induce death of immature thymocytes)[5] or due to the negative lack of a tissue-specific survival factor (eg, androgen ablation that induces death of prostatic glandular cells).[6] Once initiated, PCD leads to a cascade of biochemical and morphological events that result in irreversible degradation of the genomic DNA and fragmentation of the cell. The morphologic pathway for PCD is stereotypical and has been named “apoptosis” to distinguish this process from necrotic cell death.[2,3]

Apoptosis was originally defined by Kerr[2] as the orderly and characteristic sequence of structural changes resulting in the programmed death of the cell. The temporal sequences of events of apoptosis comprise chromatin aggregation, nuclear and cytoplasmic condensation, and eventual fragmentation of the dying cell into a cluster of membrane-bound segments (apoptotic bodies) that often contain morphologically intact organelles. For example, in apoptosis as opposed to necrotic death, mitochondria do not swell and lose their function as an early event in the process. Instead, functionally active mitochondria are often contained in apoptotic bodies, which are rapidly recognized, phagocytized, and digested by macrophages or by adjacent epithelial cells.

The period of DNA fragmentation (the F phase in the Figure) can be used to divide the temporal series of events involved in PCD.[7] During the D$_{1}$ phase of PCD, the...
cell undergoes epigenetic reprogramming in which certain genes that were previously expressed are now repressed, while other genes that were previously repressed are now expressed.[8-10] These epigenetic changes result in the activation of double-stranded DNA fragmentation during the F phase. During this F phase, the nuclear morphology changes (ie, nuclear margination of chromatin, followed by nuclear condensation), although the plasma and lysosomal membranes are still intact and the mitochondria are still functional.

Subsequent to the F phase, proteases are activated during the D phase, including the interleukin-1 beta-converting enzyme (ICE)-like proteases that hydrolyzes poly (ADP-ribose) polymerase.[11] In addition, other ICE-like proteases[12] degrade the lamins in the nuclear membrane, and the nucleus itself undergoes fragmentation. Plasma membrane blebbing and eventual cellular fragmentation into clusters of membrane-bound apoptotic bodies occurs subsequent to the nuclear fragmentation. Once formed, these apoptotic bodies are rapidly recognized, phagocytosed, and digested by macrophages or by adjacent epithelial cells.

The overall cell cycle that controls cell number is thus composed of a multicompartment system in which the cell has multiple capabilities (Figure). The cell can be (1) metabolically active but not undergoing either proliferation or death (G₀ cell); (2) undergoing cell proliferation (G₀ → G₁ → S → G₂ → mitosis), or (3) undergoing cell death by either the programmed pathway (D₀ → F → D₂ apoptotic cellular fragmentation) or the nonprogrammed (necrotic) pathway. Cells in any phase (G₀, G₁, S, or G₂ or mitosis) can be induced to enter the PCD pathway. Indeed, proliferating cells characteristically undergo programmed (apoptotic) death if their progression through the cell cycle is sufficiently perturbed.[13]

**Types of Genes Involved in PCD**

Based on the use of protein synthesis inhibitors, it is clear that the enzymatic machinery for completing PCD is already present within cells. However, numerous studies have demonstrated that the signal transduction needed to activate this pre-existing death machinery sometimes requires new protein synthesis. This suggests that there are three types of gene products involved in the process of PCD. The first are those involved in generating the signal transduction for activation of the death process in healthy, undamaged cells (sometimes referred to as physiological cell death) (Table). These type-I gene products are highly contextual with regard to the particular cell type (ie, during differentiation of the particular cell type, specific gene products are selected for such regulatory roles). Thus, the same gene product can have both the ability to stimulate cell proliferation and PCD on the differentiation status of the cell. For example, transforming growth factor-beta (TGF-beta1) is a cell-type-specific type-I gene that can stimulate proliferation of mesenchymal cells while stimulating the death of certain epithelial cells.[14] Genetic changes that lead to loss of function of these type-I genes allow the affected cells to be resistant to specific induction of physiological cell death. For example, glucocorticoids activate programmed death in certain thymus cells. If such responsive cells undergo loss of function mutations in their glucocorticoid receptor, the variant cells are resistant to glucocorticoid-induced programmed death.

The second type of PCD genes encodes proteins involved in determining the sensitivity to activation of PCD that are initiated by pathological damage to the cell (eg, radiation, viral infection, chemotherapy). This second type of PCD gene includes p53 and bcl-2. The functional expression of the gene p53 increases the sensitivity of the responding cells to activation of programmed death induced by a wide variety of damaging agents (Table). Genetic alteration leading to the loss of function of this type-II gene decreases the sensitivity of the affected cells to pathological (damage-induced) PCD (eg, from radiation, chemotherapy, viral infection). In contrast, bcl-2 is a gene whose functional expression decreases the sensitivity of the responding cells to activation of PCD induced by the same types of damaging agents. Thus, genetic alterations leading to the loss of function of this type of gene increases the sensitivity of the affected cells to pathological PCD.

Unlike the first two types of programmed death genes that encode proteins involved in the signal-transduction-induced activation of PCD, a third group of genes encode proteins that are the actual machinery needed for the process of cell death itself (Table). Genetic alteration in this third type of gene that results in loss of function would prevent the cell's ability to undergo PCD. This genotype would be reflected in a cellular phenotype that would be universally resistant to PCD induced by all treatment, both physiological and pathological. Such a universally resistant genotype would provide a great selective advantage to any cell so affected. There are cells that are resistant to hypoxia, radiation, chemotherapy, or growth factor deprivation. These observations suggest that the basic machinery to complete PCD also must be required for completion of cell proliferation. Thus, any genetic alteration in the type III PCD genes that inactivate a critical component of the machinery of PCD may lead to a sterile cell type unable to proliferate, and this genotype would be eliminated (ie, it is a lethal genotype).

**Role of Cell Proliferation in PCD**

Various independent investigators have demonstrated that proliferating cells can be induced to undergo PCD at any stage of the proliferative cell cycle (G₁, S, G₂, M). Entrance into and progression through the proliferative cell cycle is not absolutely required, however, for PCD. For example, immature thymocytes can undergo PCD when they are proliferatively quiescent (ie, out of cycle in G₀) without any attempt to enter even the earliest part of G₁.[15-17] Likewise, androgen-dependent prostatic glandular cells undergo PCD following androgen ablation in G₀ without entrance into the cell cycle.[7,18] Like normal prostate glandular cells, androgen-dependent prostatic cancer cells are induced to undergo PCD by androgen ablation, and this PCD also does not require progression into the S phase.[19]

This induction of PCD is the basis for the initial response to androgen ablation therapy that is seen in the majority of patients with metastatic prostatic cancer. Androgen ablation does not induce the programmed death of androgen-independent prostatic cancer cells. However, PCD can be induced in androgen-independent prostatic cancer cells in G₀ if the intracellular calcium is chronically sustained at twofold to threefold above baseline.[20,21]

These combined results demonstrate that PCD can be activated in both proliferation-independent (ie, G₀ or interphase death) and proliferation-dependent manners and that proliferation-dependent activation of cell death can occur at any stage of the proliferative cell cycle (Figure). In addition, although cells can undergo PCD without entrance into and progression through the S phase of the cell cycle, the double-stranded DNA fragmentation induced during PCD can activate a futile process of DNA repair.[7] Such DNA repair is not required for PCD but can lead to erroneous conclusions when using nucleotide precursor incorporation to evaluate the proliferative status of the cell undergoing PCD.

**PCD in the Prostate Induced by Androgen Withdrawal**

In the prostate, PCD is induced in the glandular epithelium following androgen deprivation. This system will be described in some detail to better illustrate the morphological, genetic, and biochemical changes that occur when a group of cells undergoes PCD in response to a specific therapy. The programmed death induced in the prostate by androgen withdrawal is cell-type specific. Only the prostatic glandular epithelial cells and not the basal epithelial or stromal cells are androgen independent and thus undergo PCD following castration.[22] These glandular cells constitute approximately 80% of the total cells in the ventral prostate of an intact adult rat and approximately 70% of these glandular cells die by seven days following castration.[22] Using the ventral prostate of the rat as a model system, the temporal sequence of events involved in the PCD pathway induced by androgen ablation has begun to be defined. After castration of these animals, the serum testosterone level drops to 1.2% of intact controls within six hours.[6] By 12 to 24 hours following castration, the level of prostatic dihydriodrostosterone (DHT), the active intracellular androgen in prostatic cells, is only 5% of intact controls. This lowering of prostatic DHT leads to changes in nuclear androgen function and at 12
During this D₁-activation phase, certain genes that were actively transcribed and translated before castration are rapidly turned off, including the C3 subunit of the prostate gene (the major secretory protein of the glandular cells), ornithine decarboxylase, histone H4, p53, and glucose-related protein.[8] Other genes that were not actively transcribed and translated become rapidly induced. These genes include c-myc,[23] glutathione S-transferase subunit Yb,[24] testosteronerepressed prostatic message (TRPM-2),[25] H-ras,[8] calmodulin,[8] TGF-beta1,[26] and tissue transglutaminase.[8] TRPM-2,[27] calmodulin,[28] and tissue transglutaminase[29] have been demonstrated to be induced in a variety of other cell types undergoing PCD. Several of these genes (c-myc, H-ras) previously have been demonstrated to be involved in cell proliferation. Comparisons of expression of these genes during castration-induced regression and subsequent androgen-induced proliferative regrowth demonstrated enhanced expression of c-myc, H-ras, and tissue transglutaminase in both prostatic cell death and proliferation.[8]

The result of this epigenetic reprogramming is that during the D₁-activation phase of the PCD process, there is a change in the profile of proteins that are synthesized, which is coupled with an inhibition of glandular cell proliferation,[30] a decrease in polyamine levels,[31] and an increase in intracellular-free Ca²⁺ levels.[32,33] The increase in intracellular free Ca²⁺ occurs following castration is derived from the extracellular pool.[32] The mechanism for this induced elevation in intracellular-free Ca²⁺ is not fully known. There are indications that enhanced expression of TGF-beta1 mRNA and protein[26] as well as the receptor for TGF-beta[126] following castration are somehow involved in the elevation of intracellular free Ca²⁺.[33]

Once the intracellular Ca²⁺ reaches a critical level, Ca²⁺-Mg²⁺-dependent endonucleases present within the nuclei of the prostatic glandular cells are enzymatically activated.[26] Normally, polyamines[34] and histone H1 maintain DNA in a compacted form that is not an efficient substrate for Ca²⁺-dependent endonucleases. However, during the D₁-activation phase there are decreases in levels of polyamines[31] and the nuclear content of histone H1[35] resulting in changes in the genomic DNA conformation and an opening up of the linker regions between nucleosomes. This enhances the accessibility of the linker DNA to the activated Ca²⁺-Mg²⁺-dependent endonuclease. Once this occurs, DNA fragmentation begins at sites located between nucleosomal units (the F phase of the PCD process (Figure) and cell death is no longer reversible.

During the F phase, the nuclear morphology changes, and there is chromatin condensation with nuclear margination even though the plasma and lysosomal membranes are still intact and mitochondria are still functional.[22] During the subsequent portion of the death process, termed the D₂ phase, the Ca²⁺-dependent tissue transglutaminase actively crosslinks various membrane proteins (J.T.I., unpublished data, 1993), with resultant cell surface blebbing, nuclear disintegration, and eventually cellular fragmentation into clusters of membrane-bound apoptotic bodies. Plasma membrane changes within these apoptotic bodies appear to be recognized by macrophages and neighboring epithelial cells; this recognition leads to rapid phagocytosis of the apoptotic bodies.[22,36,37] Thus, within seven to 10 days following castration, approximately 80% of the glandular cells die and are eliminated from the rat prostate.

**PCD Induced by Damaging Agents**

Programmed death of prostatic glandular cells following androgen ablation is an extreme case of physiological elimination of a group of cells. Normally in the adult, androgen levels are regulated such that such catastrophic elimination of prostatic cells does not occur. A low level (2% per day) of the prostatic glandular cells do undergo apoptosis, however, even in the presence of adequate androgen.[38] Presumably, this is due to the accumulation of a critical amount of damage to the cells secondary to their secretory function. These glandular cells produce and secrete large amounts of protease and polyamines,[39] both of which can be damaging to cells. If a critical amount of such endogenously generated damage is accumulated, the PCD pathway is activated. This has been demonstrated by a variety of pathological conditions, including physical agents (such as radiation, physical trauma, cold shock, and chemicals) and infectious agents (such as viruses). For example, if exposed to cold but not freezing temperatures for one to two hours, nondividing cells undergo PCD when rewarmed to 37°C.[40] With respect to ionizing radiation-induced damage, lymphocytes are the most sensitive cells in the body. Resting, nonproliferating lymphocytes rapidly undergo PCD following appropriate doses of ionizing radiation.[41] Viral infection also can induce PCD in various host target cells.[42,43] These results suggest that normal cells activate their PCD pathway when they have accumulated a critical amount of injury that cannot be adequately repaired.

PCD has been shown to occur spontaneously in a wide variety of tumor types. Apoptosis has been observed in most solid tumors including cancers of the prostate,[44] breast,[45] pancreas,[46] skin,[47] and colon,[48] as well as in several types of leukemias,[49,50] and non-Hodgkin's lymphoma.[51] Many of the commonly used chemotherapeutic agents also have been shown to cause apoptosis in chemosensitive cells. The induction of apoptosis in lymphoid tissue by glucocorticoids has been most intensively studied. In 1980, Wyllie[5] was the first to demonstrate the induction of apoptosis in immature rat thymocytes with dexamethasone treatment. Early studies in this system helped to define the classic morphological and biochemical features of apoptosis. Since these early studies, the list of agents that induce apoptosis has grown and includes alkylating agents (eg, chlorambucil and BCNU), antimetabolites (eg, fluorouracil and Ara-C), topoisomerases I and II inhibitors (eg, camptothecin and etoposide), and hormonal manipulation (eg, antiestrogens and androgen withdrawal).[52,53]

Previous studies have demonstrated that when the DNA of a proliferating cell is sufficiently damaged with a variety of chemicals, such as alkylating agents or mitogenic agents, the cell arrests in G₂, and undergoes PCD.[54] Likewise, agents such as 5-fluorodeoxyuridine that inhibit the progression through the S phase of proliferative cell cycle induce PCD.[55] Mimosine, an agent that arrests cells in the G₁ phase of the proliferative cell cycle,[56] also can induce PCD (unpublished data, J.T.I., 1993). The mechanism that enables the cell to “sense” such cellular arrest during the proliferative cell cycle and activate PCD is not understood. One possible explanation for the cytotoxic effects of cell cycle arrest is that these agents dissociate the normally integrated cell cycle events that lead to “unbalanced growth” and eventually programmed death of the cells.[13] Regardless of the mechanism, these results clearly demonstrate that PCD can be activated not only in cells in G₂ but also in cells in the various part of the proliferative cell cycle (Figure).

**Modulation of Responsiveness to PCD**

Tumor cell resistance to chemotherapy agents has emerged as an obstacle to the development of effective therapies. Mechanisms of drug resistance include drug target amplification (methotrexate), enhanced repair of DNA damage (alkylating agents), increased drug metabolism, or altered drug accumulation. Mechanisms for tumor resistance to multiple drugs include the multidrug-resistant phenotype conferred by increases in the multidrug-resistant (MDR-1) gene product encoding a plasma membrane drug efflux pump; by enhanced p53 activity; by mutations in topoisoenzymes; and by alterations in the redox detoxifying actions of glutathione.[57] Until recently, efforts to understand chemoresistance have been focused on understanding the drug/target interaction either by changes in the amount of drug present within a cell or by alterations in the amount or structure of the drug target.[58] However, a more important aspect of resistance may have less to do with the drug/target interaction and more to do with how cells sense the ensuing damage and respond to it.[57] Major questions remain unanswered: How do cells sense damage, how do they activate the PCD pathway, and how do various agents such as oncoproteins modify this sensing mechanism? Modulation of responsiveness to PCD has emerged as a new type of treatment resistance, and one that is shared by a variety of tumor types.
Susceptibility to activation of the PCD pathway varies among cell types. For instance, within the prostate, glandular epithelial cells readily undergo apoptosis (seven to 10 days) on androgen withdrawal, while neighboring stromal and basal cells are unaffected. Likewise, thymocytes rapidly apoptose when irradiated, while similar doses of radiation have no lethal effect on other cell types such as fibroblasts.[41] It is currently unknown what triggers PCD in one cell type and not another in response to the same agent. However, it appears that cells, even within the same tumor, differ in their threshold for induction of apoptosis, whether it be induced by damaging agents or by environmental changes such as growth factor deprivation. This threshold for activation of PCD has been shown to be modulated by a variety of genetic changes. For instance, loss of function of genes such as p53 or overexpression of other genes such as bcl-2 would increase the threshold for activation of PCD. Thus, cancer cells harboring these changes would become more resistant to cell-damaging agents and also would be more likely to survive removal of growth/survival factors.

New therapeutic strategies aimed at decreasing the threshold for activation of PCD (eg, by inhibiting the bcl-2 protein function) are currently under development, as well as agents capable of directly triggering the PCD cascade.

Conclusions

Several chemotherapeutic agents kill cancer cells by activation of the PCD pathway. However, in clinical practice, the majority of human tumors, especially solid tumors, remain resistant to most therapeutic agents, even when used in combination. It is becoming increasingly clear that the most important determinant of tumor resistance may be a generalized resistance to induction of PCD, rather than resistance based on specific alterations in the drug/target interaction. Alterations in a variety of oncogenes and tumor suppressor genes have been shown to modulate responsiveness to PCD induction by chemotherapeutic agents and radiation. Additional work is needed to delineate the function of these modulatory proteins and to define the cellular machinery involved in the PCD pathway itself. Further understanding of these cellular pathways should help in developing new strategies for overcoming cancer resistance and may yield more effective therapies in the future.

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References