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Role of Programmed Cell Death in Carcinogenesis

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Cells possess within their repertoire of genetic programs the ability not only to proliferate and be functionally active, but also to activate and undergo a process of self-induced destruction. This process, called programmed cell death, involves a genetic reprogramming of the cell that results in an energy-dependent cascade of biochemical and morphological changes within the cell that result in its death and elimination. Activation of this programmed death process is controlled by a series of endogenous cell-type-specific signals. In addition, a variety of exogenous cell-damaging treatments (e.g., radiation, chemicals, and viruses) can activate this pathway if sufficient injury to the cell 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 treatment that damage the cell over an extended period of time, anything that enhances the survival of initiated/damaged cells will promote the carcinogenic process. This paper presents an overview of the regulation and mechanism of programmed cell death and how derangement of this regulation may be involved in carcinogenesis.

Introduction

Major progress has been made in identifying the factors that regulate the rate of cell proliferation (e.g., *Rb*, *p53*, cyclins/*cdc* kinase). Studies have demonstrated that the proliferative cell cycle has a series of regulatory check points (*1,2*). These check points must be traversed before the cell can progress from to G_0 to mitosis (*1,2*). Until recently, it was assumed that the factors that regulate the entrance and progression through the proliferative cell cycle exclusively control cell number. This assumption is incorrect because the number of cells in a tissue, whether normal or malignant, depends on the qualitative relationship between the rate of cell proliferation and cell death. In normal adult tissues, these rates are balanced such that a steady state (i.e., self-renewing) relationship is maintained in which the number of cells in the tissue normally does not increase continuously with time. This steady state is regulated by a series of both systemic hormones and local growth factors that regulate both the rate of cell proliferation and cell death (*3*). A fundamental characteristic of cancer is that, unlike their normal counterparts, the cancer cells' rate of proliferation exceeds the rate of death, thus resulting in continuous net growth of cancer. Thus, malignant conversion results in a dysfunction in the regulation of cell proliferation and/or cell death. Regulation of cell proliferation in carcinogenesis is not the focus of this paper. Instead, the regulation and mechanism of cell death and the involvement of changes in cell death regulation in carcinogenesis is discussed.

Regulation and Mechanisms of Cell Death

Cell death can involve processes equal in complexity and regulation to those involved in cell proliferation. This has been appreciated for many years by developmental biologists. It is this group of scientists who coined the term "programmed cell death" to distinguish the active, orderly, and cell-type-specific death observed in developing organisms from necrotic cell death. Necrotic death is a response to pathological changes initiated outside of the cell and can be elicited by any of a large series of non-specific factors that result in a change in the plasma membrane permeability. This increased plasma membrane permeability results in cellular edema and the eventual osmotic lysis of the cell. In necrotic cell death, the cell has a passive role in initiating the process of cell death (i.e., the cell is killed by its hostile microenvironment). Program-

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med cell death is a process whereby a cell is activated by various signals to undergo an energy-dependent process of self-induced death. In this self-induced death, the cell is an active participant in its own demise [i.e., the cell is induced to commit "suicide" by specific signals in an otherwise normal microenvironment (*4,5*)]. Programmed cell death is a widespread phenomenon occurring normally at different stages of morphogenesis, growth, and development of metazoans (*6*). It also occurs in adult tissue (*6*). Programmed cell death is initiated in specific cell types by tissue-specific extracellular agents, generally hormones or locally diffusing chemicals. Programmed cell death can be activated either by the presence of a tissue-specific inducer [e.g., glucocorticoid induces death of small thymocytes (*7*)] or by the lack of a tissue-specific repressor [e.g., decrease in serum adrenocorticotropin hormone results in cell death in the zona reticularis of the adrenal (*8*)]. Once initiated, either by the presence of an inducer or by lack of a repressor, programmed cell death leads to a cascade of biochemical and morphological events that results in the irreversible degradation of the genomic DNA and fragmentation of the cell (*7,9–12*). The morphological pathway for programmed cell death is rather stereotypic and has been termed "apoptosis" to distinguish this process from necrotic cell death.

Apoptosis was originally defined by Kerr et al. (*4*) 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) which 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. These apoptotic bodies are rapidly recognized, phagocytized, and digested by either macrophages or adjacent epithelia cells. In programmed cell death, fragmentation of genomic DNA is an irreversible event that commits the cell to die and occurs before changes in plasma and internal membrane permeability (*5*). In many systems, DNA fragmentation has been shown to result from activation of an endonuclease present within the cell's nucleus, which selectively hydrolyzes DNA at sites between nucleosomal units, thus resulting in the stereotypic nucleosomal-sized ladder of DNA fragments (*7,9–12*). In many cell types nuclease is calcium dependent, and its activation is triggered by a sustained elevation in the intracellular free Ca^{2+} concentration initiated early in programmed cell death (*13–18*). DNA is also degraded during necrotic cell death; however, this is a late event in necrotic cells whose plasma and internal membranes have already lysed. In necrotic death, DNA is degraded into a continuous spectrum of sizes as a result of the simultaneous action of lysosomal proteases and nucleases released in dead cells (*19*).

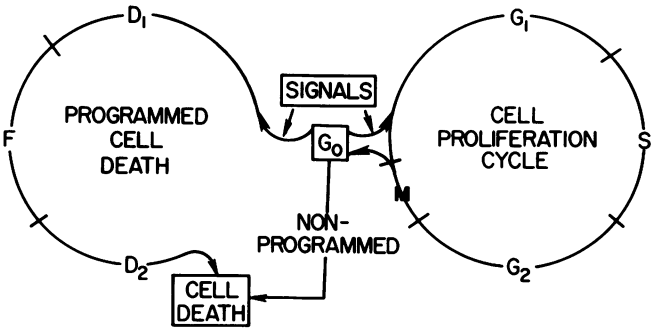

FIGURE 1. Schematic view of the cell cycle options for G_0 cell.

Thus, the period of DNA fragmentation (the F phase) can be used to divide the temporal series of events involved in programmed cell death, much as the period of DNA synthesis (the S phase) is used to divide the proliferative cell cycle. The overall cell cycle controlling cell number is thus composed of a multi-compartment system in which the cell has at least three possible options (Fig. 1). The cell can be *a*) metabolically active but not undergoing either proliferation or death (i.e., G_0 cell), *b*) undergoing cell proliferation (i.e., G_0 to mitosis), or *c*) undergoing cell death by either the programmed pathway (i.e., $G_0 \rightarrow D_1 \rightarrow F \rightarrow D_2$ apoptotic cellular fragmentation) or the nonprogrammed (i.e., necrotic) pathway. The systemic and local growth factor signals that regulate the progression within this cell cycle are cell type specific and are uniquely determined as part of the differentiated phenotype of the particular cell. Thus, the same growth factor (e.g., $TGF\beta_1$) can have either agonistic or antagonistic effects within the cell cycle for different cell types (e.g., mesenchymal versus epithelial cells). Therefore, the specific details of the regulatory pathway for the cell cycle vary between different cell types.

Cell Cycle for Prostatic Glandular Cells

The cell cycle of prostatic glandular cells will be used as an example of how the number of a specific cell type is physiologically regulated within the body. Androgen is the major systemic hormone for the prostate (*3*). The major source of androgen in the body is the testis, which synthesizes and secretes testosterone (the major physiological androgen) into the blood. The hypothalamic-pituitary-testis axis is tightly regulated so that the blood level of testosterone is maintained within a defined physiological range. Testosterone, derived from the blood, enters into prostatic cells where the 5α-reductase enzyme converts it into 5α-dihydrotestosterone [DHT (*20*)]. Due to the efficiency of this prostatic 5α-reductase activity, the DHT concentration within the prostate is 4- to 5-fold higher than testosterone, and 95% of the androgenic steroid

present within prostatic cell nuclei is DHT (*21*). In addition, the affinity of the androgen receptor is higher for DHT than for other endogenously occurring steroids (*23*). DHT is thus the major intracellular effector of androgen action within the prostate (*24*).

In the normal adult prostate, the epithelial cells are continuously turning over with time. In this self-renewing condition, the rate of prostatic cell death is balanced by an equal rate of prostatic cell proliferation such that neither involution or overgrowth of the gland normally occurs with time. If an adult male is castrated, the serum testosterone level rapidly decreases to below a critical value (*19*). As a result, the prostate rapidly involutes. This involution is due to a major loss in the epithelial, not the stromal, compartment of the prostate (*25*). The chronic requirement for androgen by the epithelial compartment is due to the fact that androgens regulate the total prostatic epithelial cell number by affecting both the rate of glandular cell proliferation and the rate of glandular cell death. Androgen does this by chronically stimulating the rate of cell proliferation (i.e., agonistic ability of androgen) while simultaneously inhibiting the rate of cell death (i.e., antagonistic ability of androgen) of the prostatic glandular epithelium (*26*).

The agonistic ability of androgen to stimulate prostatic glandular cell proliferation *in vivo* is well established. For example, if an animal or human is castrated and the prostate is allowed to involute and then the host is given sufficient exogenous androgen replacement, the prostatic glandular epithelial cells are recruited out of G_0 and into the proliferative cell cycle (*27,28*). Associated with this recruitment into the proliferative cell cycle is the enhanced expression of a series of genes including c-*fos*, c-H-*ras*, c-Ki-*ras*, c-*myc*, and b-*FGF* (*29*). This recruitment results in a proliferative response that restores the prostatic glandular epithelial cell number (*30*). Although the agonistic ability of androgen on prostatic glandular cell proliferation has been well established by a variety of studies, the additional ability of androgen to antagonistically inhibit the rate of death of these glandular cells has been fully appreciated only recently (*26,31*).

The importance of the androgenic regulation of prostatic glandular cell death is illustrated by the following observations. In the adult male rat, only 2% of the total prostatic cells die per day when the serum testosterone level is sufficient for the chronic steady-state maintenance of the gland; that is, the cell proliferation rate per day is also 2% to balance this normal rate of cell death (*26*). Within 2 hr after castration of an adult male rat, serum testosterone decreases to below 2% of the value present in the intact host (*19*). This rapid decline in serum testosterone results in the prostatic DHT concentration decreasing within the first 24 hr after castration to below a critical threshold value, triggering a cascade of events in the androgen-dependent prostatic glandular epithelial cells that eventually results in their death (*19,25,32–34*).

This pathway is activated when the extracellular level of various androgen-regulated peptide growth factors (e.g. b-fibroblast growth factor, keratinocyte growth factor, insulinlike growth factor-I, epidermal growth factor) decreases after castration to below a critical level within the prostate (*28*). The time required for the critical decrease in these growth factor levels varies regionally within the prostatic glandular cell population. The extracellular levels near some epithelial cells reach the critical value within 2 days after castration, whereas these values are not reached near other cells until 10-14 days after castration. Once this critical decrease occurs, however, a major genetic reprogramming occurs in the prostatic glandular cells, resulting in the activation phase (i.e., D_1) of the death cycle (Fig. 1).

During the D_1 phase, certain genes that were actively transcribed and translated before castration (e.g., genes for secretory proteins, polyamine synthesis enzymes, and endoplasmic reticular proteins) are rapidly turned off, while other genes which initially were not actively transcribed and translated are now rapidly induced when the program for cell death is activated. Some of the genes that are enhanced during the D_1 phase of the death cycle are c-*myc* (*35*), c-*fos* (*35*), heat shock 70 (*35*), calmodulin (unpublished observations), glutathione *s*-transferase Yb_1 (3β), α-prothymosin (unpublished observations), sulfated glycoprotein-2 (*37*), transforming growth factor β and β_3 (*34*), tissue transglutaminase (unpublished observations), and mitochondrial cytochrome C-oxidase subunit I (unpublished observations). The result of this genetic reprogramming is that during the D_1 portion of the death cycle (Fig. 1), the total level of mRNA synthesis decreases, cell proliferation stops, endoplasmic reticular volume and complexity decreases (i.e., cellular atrophy), polyamine level decreases, and intracellular free Ca^{2+} levels increase. These changes are reversible if sufficient androgen is restored.

Once the intracellular Ca^{2+} reaches a critical level, endonuclease in the cell nucleus is activated (*33,38*). The decrease in nuclear polyamine levels, coupled with a rise in the nuclear level of the highly acidic (pI = 3.5) α-prothymosin protein induces a change in the genomic DNA, opening up the linker region between nucleosomes. This enhances the accessibility of the DNA to the $Ca^{2+}Mg^{2+}$-dependent endonuclease. Once this occurs, DNA fragmentation begins (i.e., F portion of the death cycle; Fig. 1) and cell death is no longer reversible. DNA fragmentation continues until all of the genomic DNA is degraded into nucleosomal-size pieces (*19*). During this F stage, the nuclear morphology changes (e.g., chromatin condensation with nuclear margination), even though the plasma and lysosomal membranes are still intact and mitochondria are still functional. During the subsequent D_2 portion of the death cycle, Ca^{2+}–dependent tissue transglutaminase actively crosslinks various membrane proteins, and cell surface blebbing, nuclear disintegration, and eventually cellu-

lar fragmentation occur, resulting in clusters of membrane-bound apoptotic bodies. Once formed, these apoptotic bodies are rapidly phagocytized by macrophages or neighboring epithelial cells. The half-life of apoptotic bodies in the prostate induced after castration is 4-6 hrs (unpublished observations). Once ingested, the apoptotic bodies are degraded within the lysosome of the phagocytizing cells into reusable macromolecules (e.g., amino acids, nucleotides, carbohydrates).

Programmed cell death induced by androgen ablation does not require the prostate cells to undergo cell proliferation. This is demonstrated by the fact that there is no increase in the percentage of prostate cells in the S phase of the cell cycle as determined at 6 hr intervals, between 0 and 72 hr after castration, using flow cytometric methods (Berges et al., unpublished results). In contrast, the already low percentage of prostatic cells in the S phase actually decreases after castration during the period when cells are rapidly dying. This has been confirmed by pulse-chase experiments in which animals were injected with [^{3}H]thymidine 24 hr after castration and the prostates examined over the subsequent 24 hr by radioautography to determine the percentage of apoptotic bodies that are [^{3}H]thymidine labeled. These results demonstrated that at all times during the chase period, less than 10% of the apoptotic bodies are thymidine labeled, and the vast majority (>90%) of the prostatic cells that die after castration do so without entering the S phase of the cell cycle (i.e., the cells die in interphase).

Programmed Cell Death Induced by Damaging Agents

Programmed death of prostatic glandular cells after 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 programmed cell death even in the presence of adequate androgen (*26*). 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 damage cells. As discussed below, if a critical amount of such endogenously generated damage is accumulated, the programmed cell death pathway is activated. This has been demonstrated by a variety of pathological conditions, including physical [e.g., radiation, physical trauma, cold shock, chemicals, and infectious agents (e.g., viruses)]. For example, when exposed for 1-2 hrs to cold, but not freezing temperatures, nondividing cells undergo programmed cell death when rewarmed to 37°C (*40*). With respect to ionizing radiation-induced damage, lymphocytes are the most sensitive cell in the body. Resting, nonproliferating lymphocytes rapidly undergo programmed cell death after appropriate doses of ionizing radiation (*41*). Viral infection can also induce programmed cell death in various host target cells (*41–43*). These results suggest that normal cells activate their programmed cell death pathway when they have accumulated a critical amount of injury that cannot be adequately repaired.

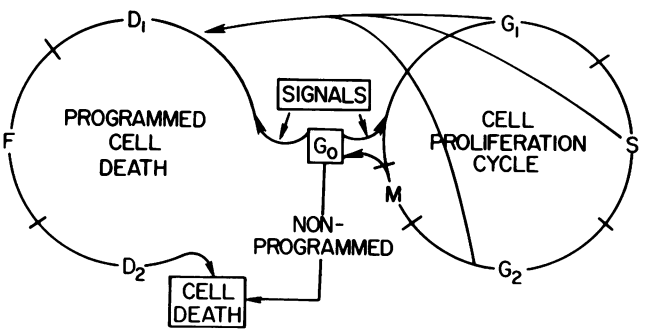

FIGURE 2. Schematic view of the cell cycle options for a cell in either G_0 or in the proliferative cell cycle.

Previous studies have demonstrated that when the DNA of a proliferating cell is sufficiently damaged with a variety of chemicals (e.g., alkylating agents, mitogenic agents, etc.), the cell arrests in G_2 and undergoes programmed cell death (*44*). Likewise, agents (e.g., 5-flurodeoxyuridine) that inhibit the progression through the S-phase of the proliferative cell cycle induce programmed cell death (*45*). Mimosine, which arrests cells in the G_1 phase of the proliferative cell cycle (*46*), can also induce programmed cell death (unpublished observations). The mechanism for how the cell senses such cellular arrest during the proliferative cell cycle and activates programmed cell death is not understood. One explanation of the cytotoxic effect of cell cycle arrest is that these agents dissociate the normally integrated cell cycle events, which leads to "unbalanced growth" and eventually programmed death of the cells (*47*). Regardless of the mechanism, these results clearly demonstrate that programmed cell death can be activated not only in cells in G_0 but also in cells in the various parts of the proliferative cell cycle (Fig. 2).

Programmed Cell Death and Carcinogenesis

The fact that potentially mutagenic/carcinogenic damage to a cell can activate its self-induced elimination via programmed death has direct implications for both the normal rate of tissue renewal and carcinogenesis. In 1982, Umansky (*48*) proposed that programmed cell death is critically involved in the process of carcinogenesis. Umansky reasoned that the frequency of mutation (i.e., 10^{-6}–10^{-9} per gene per cell generation) multiplied by the daily number of cells dividing in a human (i.e., 10^9 cell/day) should produce a large number of cells with a potential oncogene mutation (*48*). This would predict that tumor occurrence should be very high. To explain why a tumor does not always

develop in an individual, Umansky suggested that induction of programmed cell death was at least one mechanism for the elimination of cells with oncogenic mutations (*48*). This suggestion is based on the observation that most carcinogens are damaging and thus also cytotoxic, as well as transforming. Thus, the majority of cells that become initiated by carcinogens undergo programmed cell death before giving rise to progeny. Due to their death, these initiated cells are eliminated from the tissue population without increasing the risk of tumor formation. Umansky proposed in order for the cell to be transformed and give rise to a tumor, it must undergo changes that modify its responsiveness to programmed cell death so as not to be initially eliminated after exposure to the carcinogen (*48*).

Modulation of Responsiveness to Programmed Cell Death

Any mechanism that allows more carcinogen-injured cells to survive will increase the risk of developing a cancer. Also because multiple genetic changes are required for a cell to become tumorigenic, any mechanism that enhances cell survival increases the likelihood of accumulating the necessary genetic changes required for the development of a tumorigenic phenotype. In this regard it is highly significant that the Bcl-2 oncogene has been demonstrated to be oncogenic not due to an effect on cell proliferation but due to an effect on cell survival (*49,50*). Overexpression of the Bcl-2 protein occurs in human follicular B-cell lymphomas associated with a t(14;18) chromosomal translocation. Overexpression of the Bcl-2 protein extends the normal survival of B-cells by inhibiting programmed cell death and thus increasing the risk of acquisition of additional genetic changes needed for tumor progression (*49*). Additional studies have demonstrated that Epstein-Barr virus infection can likewise inhibit programmed cell death of human B-lymphocytes and that this is due to the upregulation of Bcl-2 expression in these virus-infected cells (*51,52*).

The adenovirus E1A and E1B proteins are required for oncogenic transformation of primary rodent cells. The E1A proteins alone are sufficient for induction of DNA synthesis and cellular immortalization. Complete high-efficiency transformation, however, requires both E1A and E1B gene expression (*53*). The E1B gene encodes two distance transforming proteins of 19,000 and 55,000 kDa. When expressed in the absence of the 19 kDa E1B protein, E1A proteins are acutely cytotoxic via induction of programmed cell death (*44,54,55*). E1A alone can efficiently initiate the formation of foci which subsequently undergo abortive transformation in which stimulation of cell proliferation is counteracted by cell death. Cell lines with an immortalized growth potential eventually arise with low frequency. Coexpression of the E1B 19 kDa protein with E1A is sufficient to overcome abortive retransformation to produce high-frequency transformation (*53*). These latter results demonstrate that if the rate of cell death is too high, enhancement of cell proliferation is not always sufficient for full transformation.

Similar observations have been made in studies of experimental liver carcinogenesis. Using cellular markers, it has been demonstrated that when rats are exposed to initiative doses of *N*-nitrosomorpholine, the rate of cell proliferation is much higher in preneoplastic liver foci than in the normal liver (*56*). Despite this enhanced proliferation, no net growth of the foci occurs and no liver tumors develop because the enhanced proliferation is counterbalanced by an equally increased rate of programmed cell death in these foci (*56*). During tumor promotion induced by phenobarbital (PB) treatment, a rapid net expansion of the altered foci occurs without an increase in cell proliferation. This effect is due to the PB treatment inducing a decrease in the rate of programmed cell death such that this rate is now lower than the rate of cell proliferation, thus allowing net growth of the foci into the continuously growing liver cancer.

These studies demonstrate that treatments that decrease the rate of cell death enhance the carcinogenic process. Along this line, it is significant that the state of a cell at the time it receives a potential signal to undergo programmed cell death can alter this death response. Studies have demonstrated that programmed cell death can be induced in a variety of cell types after removal of critical trophic factors and that this death response can be enhanced or inhibited depending on the status of the protein kinase C activity of the cell. For example, activation of protein kinase C with phorbol esters inhibited the programmed death of murine interleukin-2 (IL-2) dependent T-cells or murine splenic mitogen-stimulated blast cells induced by IL-2 deprivation (*57*). Similarly, the programmed cell death of murine embryo cells induced after epidermal growth factor deprivation is inhibited if protein kinase C is activated by phorbol esters (*58*). Also, the programmed death of human umbilical vein endothelia cells induced by serum deprivation was inhibited by phorbol ester treatment. Treatment of these cells with protein kinase C inhibitors induced programmed death even if the cells were maintained in the presence of appropriate growth factors (*59*). In contrast, the programmed cell death of murine thymocytes induced by glucocorticoid exposure is inhibited if protein kinase C activity is inhibited (*60*). These results demonstrate that depending on the cell type, the state of activation of protein kinase C can modify the induction of programmed cell death.

The modifying effects of protein kinase C upon cold-shock-induced programmed cell death has also been reported. When non-growing (i.e., stationary phase) hamster fibroblast cells are exposed for 1-2 hr at cold but not freezing temperatures and then rewarmed at 37°C, the cells undergo programmed cell death. In contrast, when exponentially growing hamster fibroblasts

are exposed to the cold and rewarmed, they do not undergo programmed cell death (*40*). A similar differential response to cold shock is observed between growing and stationary-phase human synovial cells (*61*). If protein kinase C is inhibited in these growing synovial cells, these cells now become sensitive to cold shock-induced programmed cell death. Likewise, if protein kinase C is activated by phorbol ester in stationary-phase synovial cells, these cells become resistant to cold shock-induced programmed cell death (*61*).

Conclusion

The results presented demonstrate that the state of the cell at the time of a potential signal to undergo programmed cell death can modify and even prevent this process. Such modification has obvious ramifications for carcinogenesis. For example, exposure to an appropriate dose of carcinogen can result in no cells being initiated in one type of tissue, but this same dose can be quite carcinogenic in cells of another organ in the same host. One explanation for this paradoxical situation could be that in transformation-resistant cells, carcinogen exposure activates programmed cell death, thus eliminating any initiated cells, whereas in transformation-sensitive cells, the state of these cells allows an occasional initiated cell to modify and thus prevent reactivation of its programmed death pathway. This would result in the survival of the initiated cell, allowing it to grow and give rise to a tumor. Such a possibility could explain the tissue specificity of particular carcinogenic treatments even though these treatments are damaging to all cell types exposed. In addition, such a possibility could explain how certain types of promoters function. Certain types of promoters (e.g., phenobarbital for liver cancers, phorbol esters for skin cancers, viral infection for certain lymphomas) could modify the response of initiated cells so that they do not complete the programmed cell death pathway. In this manner, initiated cells could survive long enough so that their progeny undergo all the genetic changes needed to acquire a tumorigenic phenotype. Major unanswered questions are how cells sense damage and activate this programmed cell death pathway and how various agents modify this sensing mechanism so that injured cells are not eliminated. These will be fruitful areas of research with regard to the role of programmed cell death in both the initiation and promotion stages of carcinogenesis.

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