# Upregulation of Apoptosis with Dietary Restriction: Implications for Carcinogenesis and Aging

S. Jill James,[1] Levan Muskhelishvili,[1] David W. Gaylor,[2] Angelo Turturro,[2] and Ronald Hart[2]

[1]Division of Biochemical Toxicology, [2]Division of Biometry and Risk Assessment, U.S. Food and Drug Administration, National Center for Toxicological Research, Jefferson, Arkansas

The maintenance of cell number homeostasis in normal tissues reflects a highly regulated balance between the rates of cell proliferation and cell death. Under pathologic conditions such as exposure to cytotoxic, genotoxic, or nongenotoxic agents, an imbalance in these rates may indicate subsequent risk of carcinogenesis. Apoptotic cell death, as opposed to necrotic cell death, provides a protective mechanism by selective elimination of senescent, preneoplastic, or superfluous cells that could negatively affect normal function and/or promote cell transformation. The relative efficiency or dysfunction of the cell death program could therefore have a direct impact on the risk of degenerative or neoplastic disease. Dietary restriction of rodents is a noninvasive intervention that has been reproducibly shown to retard tumor development and most physiologic indices of aging relative to *ad libitum*-fed animals. As such, it provides a powerful model in which to study common mechanistic processes associated with both aging and cancer. In a recent study we established that chronic dietary restriction (DR) induces an increase in spontaneous apoptotic rate and a decrease in cell proliferation rate in hepatocytes of 12-month-old B6C3F1 DR mice relative to *ad libitum* (AL)-fed mice. This diet-induced shift in cell death/proliferation rates was associated with a marked reduction in subsequent development of spontaneous hepatoma and a marked increase in disease-free life span in DR relative to AL-fed mice. These results suggest that total caloric intake may modulate the rates of cell death and proliferation in a direction consistent with a cancer-protective effect in DR mice and a cancer-promoting effect in AL mice. To determine whether the increase in spontaneous apoptotic rate was maintained over the life span of DR mice, apoptotic rates were quantified in 12-, 18-, 24- and 30-month-old DR and AL mice. The rate of apoptosis was elevated with age in both diet groups; however, the rate of apoptosis was significantly and consistently higher in DR mice regardless of age. In double-labeling experiments, an age-associated increase in the glutathione *S*-transferase-II expression in putative preneoplastic hepatocytes in AL mice was rapidly reduced by apoptosis upon initiation of DR. Thus, interventions that promote a low-level increase in apoptotic cell death may be expected to protect genotypic and phenotypic stability with age. If during tumor promotion an adaptive increase in apoptosis effectively balances the dysregulated increase proliferation, the risk of permanent genetic error and carcinogenesis would be minimized. — *Environ Health Perspect* 106(Suppl 1):307–312 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-1/307-312james/abstract.html*

Key words: apoptosis, proliferation, homeostasis, dietary restriction, cancer, aging

## Introduction

### Cell Number Homeostasis

A dynamic equilibrium exists between the opposing processes of cell birth and death in multicellular organisms to maintain constant parenchymal cell numbers throughout adult life. Cell number homeostasis depends on an integrated balance between mitosis and apoptosis such that during normal cell turnover the rates of these two processes are counterbalanced and equivalent (*1,2*). Although less appreciated, the homeostatic maintenance of this counterbalance additionally may contribute a critical defense mechanism in the response to external stressors and genotoxic and nongenotoxic agents. The increased proliferation observed in preneoplastic lesions is often accompanied by a parallel increase in apoptosis (*3,4*). In this pathologic context, the adaptive apoptotic response may serve an important protective role in countering aberrant hyperplasia. The slow-growth phenotype during tumor promotion is attributable to the adaptive upregulation of apoptosis in response to dysregulated proliferation (*5,6*). Similarly, the synchronous wave of apoptosis that follows the withdrawal of liver mitogens or trophic factors is a reflection of the homeostatic response to reestablish original cell number (*7–9*). Several recent studies have demonstrated that preexisting initiated cells are preferentially eliminated by apoptosis following withdrawal of certain tumor promoters (*10,11*). Interestingly, an increasing number of tumor promoters appears to act by inducing resistance to apoptosis in initiated cells (*12–14*). Thus, it is apparent that maintenance of cell number homeostasis is not only essential for normal physiology but additionally is an important adaptive defense mechanism against aberrant hyperplasia.

### Homeostatic Balance between Apoptosis and Proliferation Lowers Risk of Cell Transformation

Tissue hypertrophy or atrophy is the direct result of chronic imbalance in rates of cell proliferation and death (*2*). The loss of homeostatic balance is an underlying factor in the pathogenesis of numerous human disease states, including cancer. Although both proliferation and apoptosis are elevated in preneoplastic lesions, a marginal imbalance favoring proliferation is reflected by the slow-growth phenotype. The complementary increase in apoptosis that accompanies proliferative stimuli ensures that the risk of unrepaired DNA polymerase errors does not go unchecked. At this stage, although proliferation is increased, it is not uncontrolled (*15,16*). By the preferential elimination of initiated or preneoplastic cells, apoptosis effectively attenuates the risk of cell transformation due to proliferation-related mutagenesis. Although increased proliferation clearly is necessary, it may not be independently sufficient for cell transformation, as recently suggested by Farber (*17*).

This paper is based on a presentation at The Third BELLE Conference on Toxicological Defense Mechanisms and the Shape of Dose–Response Relationships held 12–14 November 1996 in Research Triangle Park, NC. Manuscript received at *EHP* 7 March 1997; accepted 22 May 1997.

Address correspondence to Dr. S.J. James, U.S. Food and Drug Administration, National Center for Toxicological Research, Division of Biochemical Toxicology, 3900 NCTR Road, Jefferson, AR 72079. Telephone: (870) 543-7306. Fax: (870) 543-7720. E-mail: jjames@nctr.fda.gov

Abbreviations: used: ABs, apoptotic bodies; AL, *ad libitum*; DR, dietary restriction; GST, glutathionine *S*-transferase; IGF, insulinlike growth factor; PCNA, proliferating cell nuclear antigen; TUNEL, TdT-mediated dUTP-digoxigenin nick end-labeling.

Recent evidence suggests that the transition between tumor promotion and tumor progression ultimately may depend on the failure of apoptosis in the presence of dysregulated proliferation; i.e., both aberrations are essential for cell transformation (*18*). The importance of proliferation as a major risk factor may only become significant when it is liberated from the constraints imposed by apoptosis, i.e., when it becomes truly uncontrolled. Positive and negative regulation of apoptosis occurs by *a*) induction of signals leading to pathways of cell death and/or increasing cell sensitivity to cell death and *b*) inhibition of one or more of the several pathways leading to an irreversible commitment to cell death. Genetic or environmental perturbations that lead to pathologic dysregulation of these inducers and inhibitors of apoptosis disrupt the dynamic balance between proliferation and cell death.

Thus, permanent loss of homeostatic equilibrium between cell proliferation and death may be a critical determinant in the transition from the reversible stage of tumor promotion to the irreversible commitment to tumor progression. Once apoptotic competence is lost, the heritable oncogene-activated proliferative advantage effectively precludes recovery of cell number homeostasis and a point-of-no-return establishes the malignant state. These relationships were recently confirmed in a mouse model of multistage tumorigenesis of islet β cells (*19*). In this study the incidence of apoptosis was increased in parallel with increasing proliferation during tumor promotion; however, the transition to malignancy was associated with a dramatic drop in apoptotic rate with no further change in proliferation rate.

Although early attempts to understand the mechanistic basis of the carcinogenic process initially focused on increased proliferation and the associated increased risk of mutation as the major risk factor, recent emphasis has shifted to inhibition or resistance to apoptosis as the pivotal determinant in cell transformation (*20*). Given the homeostatic yin/yang responsiveness between cell proliferation and cell death, the two processes are, in fact, inseparable for the understanding of both normal physiology and pathologic processes. It is becoming increasingly clear that dysregulation in one process is only meaningful in the context of the other. Thus, it may not be the rare cell with a proliferative advantage that becomes transformed but the rare cell with proliferative advantage that additionally acquires apoptosis resistance.

It should be noted that the adaptive increase in apoptotic rate that accompanies dysregulated proliferation during tumor promotion may act as a double-edged sword by increasing the selection pressure for apoptosis resistance. Because the apoptotic cascade involves endonuclease-mediated DNA strand breaks, it is conceivable that errors in DNA synthesis associated with strand break repair in the early stages of apoptosis could result in point mutations with survival advantages. Unrepaired DNA strand breaks can promote DNA lesions such as base deletions and frameshift errors that could inactivate genes involved in the apoptosis pathway (20). In addition, nonmutagenic mechanisms can promote apoptosis resistance. These include hypomethylation and overexpression of apoptosis-regulating genes such as *bcl*-2 (*21*) and/or loss of gap junctional communication (*22*). Although most experimental evidence points to increased proliferation as the primary stimulus for the compensatory increase in apoptosis, recent reports suggest that a primary increase in apoptosis can also stimulate compensatory proliferation (*23,24*). These results would suggest a bidirectional responsiveness to either increased apoptosis or to increased mitosis.

The interactive responsiveness between cell birth and death implies that regulatory gene coordination must exist between the two signal transduction pathways (*25,26*). Consistent with this possibility, several cellular proteins including c-myc, p53, E2F, and the viral protein E1A have dual functions as positive regulators of both cell proliferation and cell death, with the final outcome dependent on the local micro-environment (*15,27*). The retinoblastoma tumor suppressor gene product pRb may function as a negative regulator of both proliferation and apoptosis by binding with c-myc, E2F, and E1A (*28*). Gene products conferring resistance to apoptosis include bcl-2, bcl-xl, c-abl, and insulinlike growth factor (IGF)-II (*15,29,30*). Overexpression of these genes appears to block a late-stage step in the apoptotic pathway and permits cell survival without stimulation of proliferation. Finally, the mutated form of the oncogene H-*ras*, a well-established enhancer of proliferation, recently has been implicated as a repressor of apoptosis (*29,30*). Continued research on these common pathways should further confirm multiple levels of coordinated gene interaction in the homeostatic control of cell numbers.

## Dietary Restriction, Apoptosis, and Cell Number Homeostasis

Dietary restriction (DR) is a well studied intervention that retards tumor development and extends maximum life span relative to *ad libitum* (AL)-fed animals (*31–34*). Cancer incidence increases progressively with age; thus, the hormonal and metabolic alterations associated with chronic DR provide a model system in which to study mechanistic processes common to both cancer and aging (*35*).

Because apoptosis appears to preferentially eliminate preneoplastic, senescent, or DNA-damaged cells, agents, or conditions that promote apoptosis would be expected to negatively affect tumor development (*10*). In a recent series of studies, we tested the hypothesis that a relative increase in the basal rate of apoptosis with DR may contribute to the mechanisms of tumor resistance and prolongation of life span by promoting natural selection at the cellular level. Spontaneous rates of apoptosis and proliferation were quantified in liver sections from 12-month-old DR and AL-fed male B6C3F1 mice, a murine strain with increased genetic susceptibility to developing spontaneous liver tumors by 18 months of age (*36*). The DR group received 60% of the AL consumption of an NIH-31 open-formula diet supplemented with vitamins to ensure equal micronutrient consumption between groups. The 12-month-old age group was selected for immunohistochemical analysis because it represents a preneoplastic stage at which evaluation of cell proliferation and cell death rates may be predictive of subsequent tumor development. To ensure that animals from both dietary groups were metabolically and hormonally synchronized in terms of food-induced circadian rhythms, mice from both groups were fasted for 24 hr before being euthanized at the same time of day.

Visualization of apoptotic bodies (ABs) was facilitated by TdT-mediated dUTP digoxigenin nick end-labeling (TUNEL) using the Apoptag detection system (Oncor, Gaithersburg, MD). In Figure 1, a representative liver section demonstrates the utility of the TUNEL method to facilitate the identification of ABs. Two hundred fields (~50,000 hepatocytes) per animal were counted and the apoptotic activity expressed as the number of ABs per 100 hepatocytes (percentage incidence). Proliferating hepatocytes in S phase were quantified by immunohistochemical analysis of proliferating cell nuclear antigen

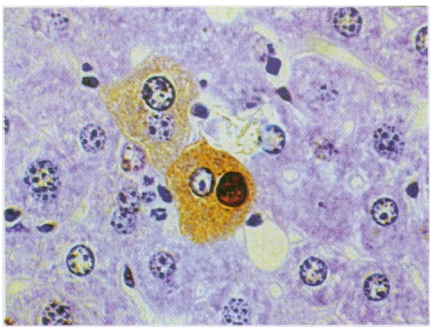

**Figure 1.** Representative example of immunohistochemical staining of a phagocytized apoptotic body in a liver section from a diet-restricted B6C3F1 mouse. Sample counterstained with methyl green (×340).

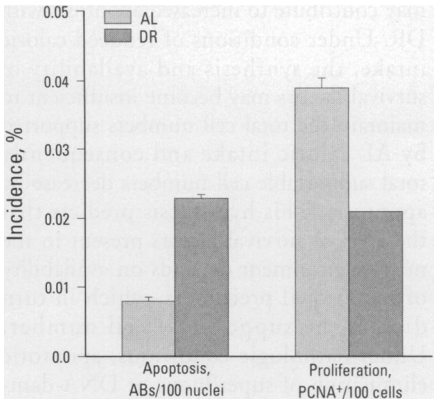


**Figure 2.** Comparison of the rates of apoptosis (apoptotic bodies/100 nuclei) and proliferation (PCNA-positive cells/100 hepatocytes) in livers of 12-month-old AL-fed and 40% DR B6C3F1 mice. Note relative increase in incidence of apoptotic bodies and decrease in PCNA-positive nuclei in livers of DR mice. Data are the means ± SEM for the 14 mice in each dietary group.

(PCNA) using a standard biotin–avidin peroxidase detection system. Results were expressed as the relative incidence of PCNA-positive cells in 200 fields (50,000 hepatocytes). Because of the very low frequency of ABs in normal liver, 15 mice per group were evaluated to increase the precision of the mean.

Both apoptotic and proliferative activity in liver sections from 12-month-old DR and AL-fed mice are presented in Figure 2. The increase in the spontaneous level of hepatocyte apoptosis and decrease in proliferative activity in the DR mice relative to those in AL-fed mice were associated with a significantly lower incidence of spontaneous hepatoma over a 36-month period, as shown in Table 1. The results suggest that caloric intake may modulate the basal turnover rates of cell death and birth in a direction consistent with a cancer-protective effect in the DR mice and a cancer-promoting effect in AL-fed mice. An increase in the elimination of potentially neoplastic, diseased, or damaged cells by apoptosis in the DR animals would effectively create a cohort of healthier, more robust cells with improved functional status relative to that in the AL-fed counterparts. This natural selection (survival of the fittest) at the cellular level would tend to promote a disease-free increase in life span that has been documented to be associated with DR in a variety of mammals and multicellular organisms.

Despite the differences in rates between proliferation and cell death with DR and AL feeding, tissue-size homeostasis (liver weights within groups and liver weight/g body weight between groups) was remarkably constant over the life span of the mice, as indicated in Table 2. Therefore, we postulate that low-level or single-cell necrotic cell death must occur in livers of AL-fed mice such that total cell death and proliferation are in approximate balance. Although difficult to quantify, single cells exhibiting necrotic morphology such as swelling, blebbing of cytoplasmic membrane, and nuclear pycnosis were histologically visible in liver sections from AL-fed mice and were nonexistent in livers of DR mice. An increase in necrotic cell death in AL mice would explain the constant liver weights despite a decrease in apoptotic cell death. This is an important distinction because necrotic cell death is considered to be a pathologic response to severe toxicologic insults not associated with the active and preferential elimination of initiated or preneoplastic cells (*7*).

**Table 1.** Numbers of B6C3F1 mice with hepatocellular tumors as a function of age and diet ($n$=14 mice/interval/group).

| | Age, months | | | | |
|---|---|---|---|---|---|
| Dietary group | 12 | 18 | 24 | 30 | 36 |
| AL | 0 | 4 | 3 | 5 | 8 |
| DR[a] | 0 | 0 | 0 | 3 | 0 |

[a]40%.

## Dietary Restriction, Aging, and Apoptosis

To determine whether the increase in spontaneous apoptotic rate was maintained over the life span of DR mice, we quantified apoptotic rates in 12-, 18-, 24-, and 30-month-old DR and AL B6C3F1 mice (*37*). The rate of apoptosis was elevated with age in both diet groups; however, the rate of apoptosis was significantly and consistently higher in the DR mice regardless of age. It has been speculated that in addition to preferential elimination of preneoplastic cells, apoptosis provides a cellular defense mechanism to subvert the tendency of senescent cells to undergo transformation (*38*). Thus, the increase in spontaneous apoptotic activity in the livers of aging DR B6C3F1 mice may contribute to the mechanism of tumor resistance and life span extension.

In a third study liver sections from aging AL-fed and DR B6C3F1 male mice were evaluated immunohistochemically for pi-class glutathione *S*-transferase (GST)-positive putative preneoplastic cells (*39*). A progressive increase in GST-II labeling occurred from 18 to 36 months of age in the AL-fed mice and was associated with a high incidence of GST-II-positive spontaneous liver tumors. Expression of GST-II was negligible in DR mice in all age groups and was associated with a significant decrease in tumor incidence. To determine whether DR induces apoptosis in GST-II-positive hepatocytes, 24-month-old AL-fed mice were introduced to 40% DR. After 1 week of restriction a decrease in GST-II expression was associated with a 3-fold increase in frequency of apoptotic bodies, as detected by *in situ* TUNEL assay

**Table 2.** Changes in liver weights and body weights of B6C3F1 mice with age and diet.[a]

| | Age | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 12 months | | 18 months | | 24 months | | 30 months | |
| | AL | DR | AL | DR | AL | DR | AL | DR |
| Body weight, g | 38.3 ± 0.29 | 26.5 ± 0.14 | 37.9 ± 0.33 | 26.1 ± 0.14 | 39.3 ± 0.32 | 27.4 ± 0.17 | 32.3 ± 0.39 | 23.5 ± 0.10 |
| Liver weight, g | 1.628 ± 0.018 | 1.070 ± 0.064 | 1.666 ± 0.013 | 1.142 ± 0.006 | 1.593 ± 0.018 | 1.188 ± 0.013 | 1.477 ± 0.020 | 1.059 ± 0.007 |
| Liver weight/body weight | 0.042 ± 0.0002 | 0.040 ± 0.0001 | 0.044 ± 0.0002 | 0.043 ± 0.0002 | 0.041 ± 0.0002 | 0.043 ± 0.0003 | 0.045 ± 0.0002 | 0.045 ± 0.0003 |

[a]Values are the means ± SEM in nontumor-bearing mice.

(Figure 3). Double labeling of apoptotic hepatocytes revealed that about 70% of apoptotic bodies were GST-II positive (Figure 4). These results suggest that spontaneous, potentially preneoplastic hepatocytes in tumor-prone B6C3F1 mice are eliminated by apoptosis upon initiation of DR. Thus, we may tentatively conclude that DR and interventions that increase cellular sensitivity to apoptotic cell death may protect genotypic and phenotypic stability with age.

## Dietary Restriction, Survival Factors, and Apoptosis

It has been proposed that the genes for activation of the apoptotic pathway are programmed into each cell as a default mechanism that is normally and actively suppressed by the presence of essential survival factors in the microenvironment (*40,41*). Initiation of apoptosis as a result of reduced availability of survival factors may contribute to increased apoptosis with DR. Under conditions of reduced caloric intake, the synthesis and availability of survival factors may become insufficient to maintain the total cell numbers supported by AL caloric intake and consequently total supportable cell numbers decrease by apoptosis. This hypothesis predicts that the level of survival factors present in the microenvironment depends on availability of nutritional precursors, which in turn dictate the supportable cell number. Under pathologic conditions, apoptotic elimination of superfluous or DNA-damaged cells may be the most energetically efficient alternative for the calorically restricted animal.

Although the mechanistic basis for the divergent effects of caloric intake on proliferation and apoptosis is not known, the expression of the IGF-I receptor and its ligands (insulin, IGF-I, and IGF-II) are potential candidates because they clearly are modulated by caloric intake. DR in rodents reduces circulating levels of insulin, IGF-I, and IGF-II (*42*), whereas AL feeding has been associated with insulinemia (*43*). These mitogenic factors are involved in normal liver growth and, importantly, function as survival factors that confer resistance to apoptosis both *in vitro* and *in vivo* (*41,44*). A decrease in IGF-1R levels has been associated with increased apoptosis and inhibition of tumorigenesis (*45*). Studies are now in progress to correlate alterations in IGF family members with the incidence of apoptosis in the DR model.

## Caloric Restriction, Apoptosis, and Hormetic Effects

In studying the toxicologic implications of adaptive biologic responses, it is useful to consider the spectrum of caloric intake in terms of positive and negative physiologic effects. A U-shaped curve can be constructed in which both insufficient and excess energy intake are associated with negative physiological effects, as indicated in Figure 5. Between these two extremes, a range of restricted caloric intake has been associated with positive (hormetic) effects such as increased longevity (*32,46*) and reductions in tumorigenesis (*31,36*), autoimmune disease (*47,48*), and oxidative damage (*49,50*). Similarly, a U-shaped curve over the range of apoptotic response can be created. A low-level chronic increase in the background level of apoptosis would be protective (*32,35*), whereas excessive cell death would negatively affect total cell numbers and functional capacity (*51*). Negative effects such as insufficient apoptotic cell death or induction of resistance to apoptosis would also be incurred at the opposite extreme. Thus, agents or conditions that lower apoptotic activity below the protective

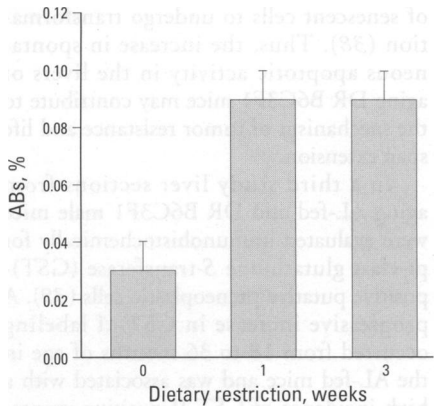


**Figure 3.** The increase in apoptotic activity after initiation of 40% DR in 24-month-old B6C3F1 mice. Data are the mean ± SEM from four animals ($p < 0.01$, AL vs DR).

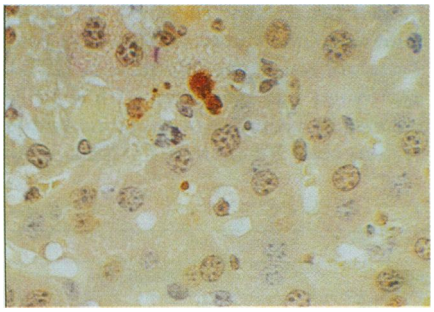

**Figure 4.** Representative example of GST-II-positive apoptotic bodies in liver section from a 24-month-old mouse 1 week after initiating DR. Alkaline phosphatase stain is red (×700).

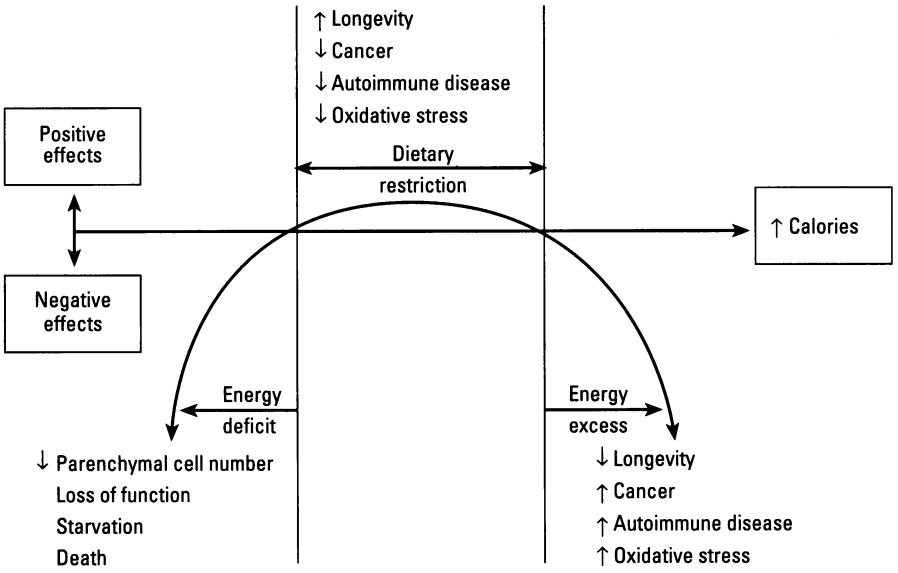


**Figure 5.** Hypothetical U-shaped curve over the spectrum of caloric intake from insufficient to excessive calories, emphasizing negative physiologic effects at both extremes and positive or hormetic effects within a range of restricted caloric intake.

threshold would tend to accelerate the accumulation of age-associated genetic lesions and neoplasia. These relationships are diagrammed as a hormetic U-shaped curve in Figure 6, with positive, cancer-protective effects occurring in the range of cell number homeostasis.

## Conclusion

The homeostatic balance between cell proliferation and apoptosis in the maintenance of constant cell numbers may provide a hormetic effect by minimizing the consequences of proliferation-related mutagenesis during tumor promotion. The adaptive increase in apoptosis that accompanies the oncogene-activated dysregulation in proliferation selectively eliminates potentially preneoplastic cells in hyperplastic foci. Acquired resistance to apoptosis appears to be a pivotal event in the transition to malignancy.

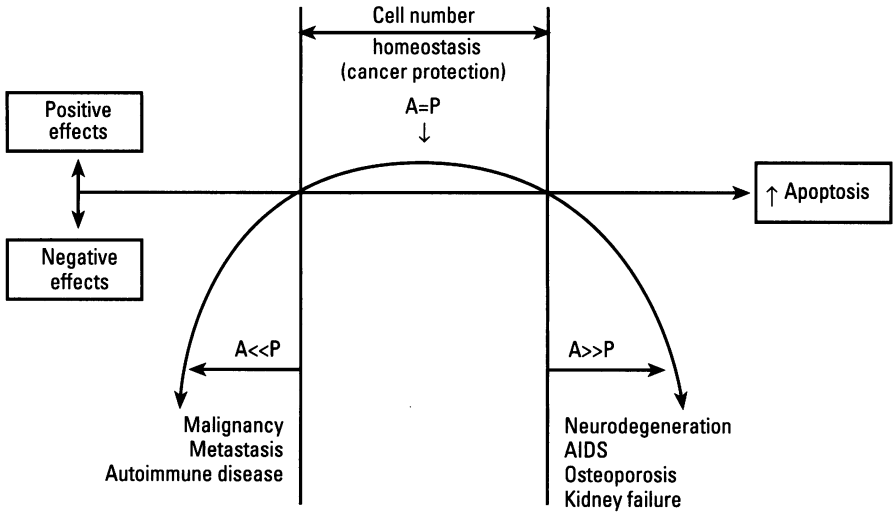


**Figure 6.** Hypothetical U-shaped curve over the range of apoptotic activity demonstrating negative effects when the rate of apoptosis (A) is much less than the rate of proliferation (P) and also when the apoptosis rate chronically exceeds the proliferation rate. Within the range of cell number homeostasis, when rates of apoptosis and proliferation are in equilibrium, the risk of neoplasia is minimized.

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