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Correlation of genetic instability and apoptosis in the presence of oncogenic *Ki*-Ras

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Abstract

The product of the *ras* proto-oncogene has been implicated as an essential signal transducer, involved in a variety of biological or pathological activities, including apoptosis. The aim of this investigation was to further explore the mechanisms of apoptosis triggered by Ras. Stable expression of constitutively-activated (*v*-)*Ki*-Ras in Balb/c-3T3 mouse fibroblasts resulted in a loss of G_1 arrest in response to treatments which induced cell cycle arrest in the parental Balb/c-3T3 cells, accompanied by decreased expression of the p53 tumor suppressor protein and the *GADD45* gene, the product of which is involved in DNA repair, and deregulated expression of the MDM-2 gene, the product of which can regulate p53 expression. *Ki*-Ras expression also increased the frequency of PALA-selectable *CAD* gene amplification, and paradoxically the susceptibility to PALA-induced apoptosis. After persistent serum-starvation, cells expressing the activated *ras* gene lost clonogenic potential, indicating impaired capability for genetic repair in the cells. Taken together, these data suggest that activated *Ki-ras* may confer genetic instability upon cells, possibly through interference with tumor suppressors, such as p53. While this instability may facilitate adaptation to environmental stresses, this instability in the genome also renders cells containing activated *ras* genes intrinsically more susceptible to programmed cell death, possibly by accumulation of undesirable or lethal genetic events during the process of tumor development.

Keywords: *ras* oncogene; p53; genomic instability; *CAD* gene amplification; clonogenicity; apoptosis; tumor suppressors

Abbreviations: PALA, N-phosphonacetyl-L-aspartate; DME, Dulbecco Modified Eagle Medium; PE, planing efficiency; MEF, mouse embryo fibroblast

Introduction

The members of the *ras* gene family, *Ha-ras*, *Ki-ras* and *N-ras*, encode 21 kDa guanine nucleotide-binding proteins. Ras proteins have been found to be involved in the regulation of many cellular biological or pathological processes, such as proliferation and differentiation, as well as apoptosis (Barbacid, 1987; Bourne *et al*, 1990; Downward *et al*, 1990; Li *et al*, 1992; Marshall, 1993; Chen and Faller, 1995, 1996). Although the direct targets of Ras proteins are not entirely defined, many downstream effectors of the Ras signaling pathway have been suggested (Hattori *et al*, 1992; Zhang *et al*, 1992; Buday and Downward, 1993; Medema *et al*, 1993; Votjtek *et al*, 1993; Harrington *et al*, 1994). Ras proteins can be activated in response to a wide variety of mitogenic or activation stimuli (Avruch *et al*, 1994; McCormick, 1994), and suppression of Ras activity using either dominant-negative mutants of Ras or neutralizing anti-Ras antibodies efficiently blocks entry of cells into S-phase after growth factor stimulation (Moodie *et al*, 1993; Khosravi-Far and Der, 1994; Winston *et al*, 1996). Recently, many studies have suggested that expression of oncogenic Ras may be associated with multiple genetic alterations which contribute to initiation of abnormal karyotype, deregulation of cell growth, development of cancer, and perhaps susceptibility to apoptosis (Hirakawa and Ruley, 1988; Li *et al*, 1992; de Vries *et al*, 1993; Denko *et al*, 1994; Arber *et al*, 1996; Chen and Faller, 1996; Chen, *et al*, 1998). However, the mechanisms by which oncogenic Ras induces these seemingly disparate genetic alterations remain poorly understood.

One of the early and common characteristics of tumor cells is genetic instability. The reconstructed genome arising during tumor development allows cells to escape from normal restriction against unlimited proliferation and metastasis (Hartwell and Weinert, 1989; Hartwell, 1992; Hattori *et al*, 1992; Hartwell and Kastan, 1994; Sherr and Roberts, 1995). Studies in yeast have demonstrated that genetic alternations in DNA damage repair processes, in the machinery for DNA replication, and in cell cycle controls, all contribute to genomic instability (Hartwell, 1992). Mutations in the tumor suppressor gene encoding p53 is the most common event in human malignancies and occurs in at least 50% of human cancers (Hartwell, 1992; Harris and Hollstein, 1993). A number of properties of wt-p53 related to its growth-regulatory function, inducing site-specific transcriptional transactivation, are also associated with its tumor-suppressive functions. For example, wt-p53 can reverse the transformed phenotype when cotransfected into primary rat embryonic fibroblasts with various combinations of oncogenes, such as *ras* plus *myc*, *ras* plus adenovirus E1A, E1A plus E1B, and *ras* plus human papillomavirus E7 (Storey *et al*, 1988; Eliyahu *et al*, 1989; Finlay *et al*, 1989; Phelps *et al*, 1989). wt-p53 is involved in the regulation of cell cycle checkpoints, especially G1/S

transition. Disruption of such checkpoints would impair timely repair of cellular or DNA damage, and contribute to the potential loss of genomic material (Kastan *et al*, 1992; Marx, 1994; Wang *et al*, 1995). Inactivation of wt-p53 in various types of cells has been demonstrated to accompany an increase incidence of gene amplification (for example *CAD* gene amplification), and such gene amplification is an indicator of the unstable genomic status of these cells (Livingstone *et al*, 1992; Yin *et al*, 1992; Carder *et al*, 1993; Schaefer *et al*, 1993; Lee *et al*, 1994; Bouffler *et al*, 1995; Donehower *et al*, 1995). As a transcription factor, wt-p53 appears to control the expression of many genes, including a repair-related gene *GADD45* (Kastan, *et al*, 1992). *GADD45* gene was found to be rapidly induced by DNA-damaging agents or other conditions eliciting cell growth arrest, such as serum starvation, in a wide variety of mammalian cells expressing wt-p53, and the *GADD45* gene product may participate in the process of cellular or DNA damage repair.

Normal or untransformed cells, such as fibroblasts and lymphocytes, arrest in the G1/G0 phase of the cell cycle in response to serum-depletion, inhibition of PKC activity, or exposure to PALA (N-phosphonacetyl-L-aspartate), an inhibitor of purine metabolism. During serum or mitogen withdrawal, constitutively-expressed c-*myc* may inappropriately drive cells into cell cycle and initiate the apoptotic process (Askew, *et al*, 1991; Shi, *et al*, 1992; Evan, *et al*, 1992). Oncogenic Ras can trigger cells to undergo apoptosis following downregulation of PKC activity (Chen and Faller, 1995). Recent studies have show that expression of activated Ras in fibroblasts or lymphocytes results in elevation of certain G1 cyclins, and subsequently alters cell cycle progression (Filmus *et al*, 1994; Lovec *et al*, 1994; Liu *et al*, 1995; Arber *et al*, 1996; McKenna *et al*, 1996; Winston *et al*, 1996). Overexpression of p16, a cyclin inhibitor, blocks Ras-mediated proliferation and the transformation caused by introduction of activated *ras* genes into primary murine fibroblasts lacking p16 (Serrano *et al*, 1995, 1997).

To further study the function of oncogenic Ras in the regulation of cell proliferation and cell death, we have used early passage mouse Balb/c-3T3 fibroblast lines which were stably-expressing a *v-Ki-ras* gene (designated *Ki*-Balb cell lines) to seek correlations among the expression of the tumor suppressor p53, genetic instability induced by Ras, and the susceptibility to programmed cell death conferred by oncogenic Ras. We show in this report that, in the presence of activated *Ki*-ras, cells cannot arrest in the G1-phase of cell cycle in response to treatments which induce cell cycle arrest in the parental Balb/c-3T3 cells. This loss of a cell cycle checkpoint is accompanied by decreases in the expression of the p53 protein and the *GADD45* gene. Analysis of *CAD* (trifunctional enzyme carbamoyl-P synthetase, aspartate transcarbamylase, dihydro-orotase) gene amplification and analysis of cell viability in the presence of PALA revealed that activated *Ki*-ras increased both the frequency of *CAD* gene amplification and the susceptibility of these cells to apoptosis. Clonogenicity assays demonstrated that cells expressing *v-Ki-ras* lost the capability to propagate following periods of serum-depletion. The combination of serum withdrawal and PALA exposure abolished the ability of *Ki*-Balb cells to form PALA-resistant colonies and increased their susceptibility to PALA-induced apoptosis. These studies provide a framework for understanding the correlation between multiple genetic changes in the presence of the *ras* oncogene and the susceptibility to apoptosis under certain environmental conditions.

Results

Oncogenic *Ki*-Ras prevents G_1 arrest during serum-depletion

Activated *(v-) Ki-ras* were introduced into murine Balb/c-3T3 fibroblast cell lines stable-infection (designated *Ki*-Balb cell lines). To assess cell cycle regulation in the presence or absence of serum in *Ki*-Balb cells in comparison to the parental Balb-3T3 cells, and to investigate the potential role of activated *Ki*-Ras in this process, nuclear DNA profiles of Balb and *Ki*-Balb cells, growing in medium containing either 10 or 0.05% of newborn calf serum for 48 h, were examined (Figure 1). After 48 h of serum-starvation, the majority of Balb-3T3 cells were arrested in the G0/G1-phase. A significant proportion of *Ki*-Balb cells, however, after 48 h of serum-starvation, remained in the S and G2/M-phases, although the proportion of actively growing cells (S+G2/M) was reduced in comparison with the same cells cultured in medium containing 10% serum. These results indicate that there is a disruption of growth arrest in cells expressing *Ki*-Ras cells after serum-depletion.

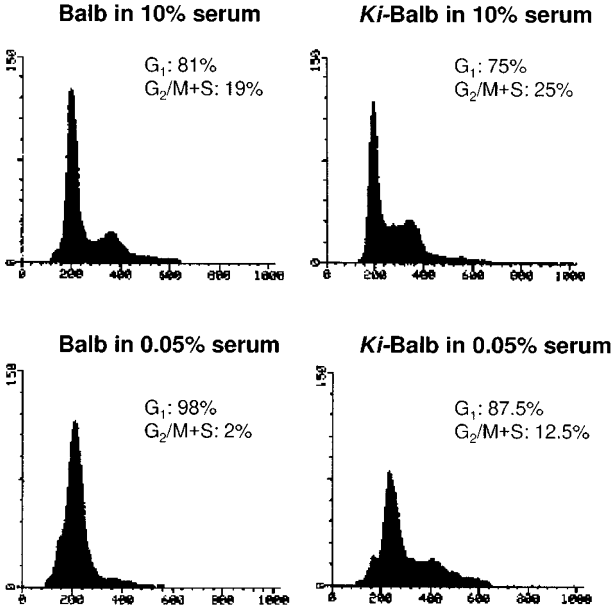


Figure 1 DNA profiles of Balb fibroblasts, with or without oncogenic *Ki*-Ras under normal growth conditions or after 48 h of serum-depletion. The percentages of cells in G1, S, and G2/M are indicated

Expression of wt-p53 is decreased in cells expressing *Ki*-Ras

As p53 has been implicated in the regulation of cell cycle arrest and apoptosis (Livingstone *et al*, 1992; Vogelstein and Kinzler, 1992; Yin *et al*, 1992; Prives, 1993, 1994; Moll *et al*, 1996), the expression of wt-p53 protein in the nuclear-free cell lysate fraction and in the nuclear fraction of Balb and *Ki*-Balb cells was examined under normal growth conditions or after serum-starvation for 48 h (Figure 2a). Equal amounts of proteins from either the nuclear-free cell lysate or the nuclear fraction of the two cell types were immunoprecipitated, and subsequently immunoblotted, with an anti-wt-p53 antibody. wt-p53 protein was detectable in both subcellular fractions of Balb cells under normal growth conditions (Figure 2a, lanes 1 and 5), and was increased (by 2–2.8-fold) in both fractions after 48 h of serum-starvation (Figure 2a, lanes 2 and 6). wt-p53 protein in the nuclear-free cell lysate fraction or in the nuclear fraction of *Ki*-Balb cells was almost undetectable under normal growth conditions (Figure 2a, lanes 3 and 7). Following 48 h of culture in serum-depleted medium, there was only a minimal and non-significant increase (less than 0.5-fold) in the level of wt-p53 protein in the cell lysate (Figure 2a, lane 4), and no change in the nuclear fraction (Figure 2a, lane 8) in *Ki*-Balb cells. The same phenomenon has been observed in a human T lymphoblastoid cell line, Jurkat. wt-p53 protein is undetectable in Jurkat cells expressing activated Ras during normal growth or after exposure to agents capable of inducing G1 arrest in parental Jurkat cells (data not shown). A number of different *Ki*-Balb clonal cell lines and pools were tested for wt-p53 dysregulation, growth properties, sensitivity to serum-deprivation, clonogenicity and PALA sensitivity (as described below), and all had identical phenotypes.

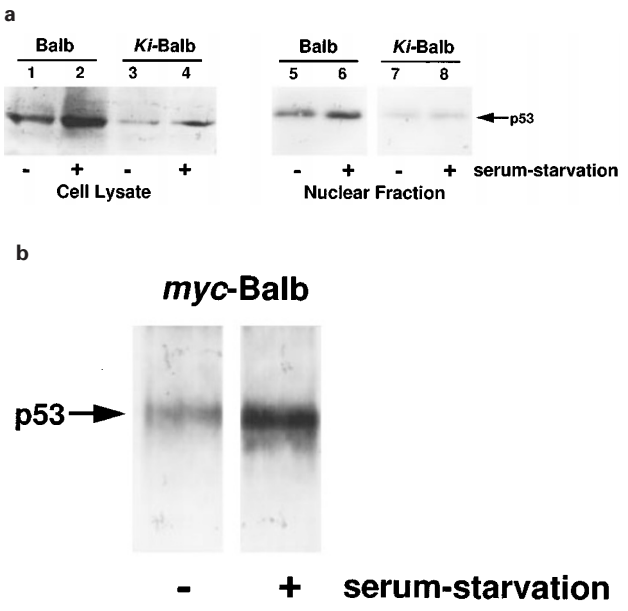


Figure 2 The expression of wt-p53 protein with or without expression of an activated *ras* or *myc* gene following serum-starvation or under normal growth conditions. (**a**) The nuclear-free cell lysates or nuclear extracts of Balb and *Ki*-Balb cells, under conditions with (+) or without (−) serum-starvation were immunoprecipitated and subsequently immunoblotted for wt-p53 expression. (**b**) The cell lysates of Balb cells transfected with oncogenic *myc* in the presence (+) or absence (−) of serum-starvation were immunoprecipitated and then immunoblotted for wt-53 expression

It is normally necessary to introduce two complementing oncogenes, such as *myc* and *ras*, to efficiently transform primary cells, and forced overexpression of c-Myc has been shown to promote apoptosis under certain conditions (Askew, *et al*, 1991; Shi, *et al*, 1992; Evan, *et al*, 1992). To determine if *myc* alone can also suppress the expression of wt-p53 protein, Balb cells were stably-transfected with the *myc* proto-oncogene (*myc*-Balb cells) and the levels of wt-p53 protein were examined in the cell lysates under normal growth conditions or after serum-starvation (Figure 2b). There was a low level of wt-p53 protein under normal growth conditions in the *myc*-Balb cells and the p53 protein level was dramatically increased after 48 h of serum-starvation (>threefold). Yet, like the cells expressing activated Ras, the *myc*-Balb cells were not arrested in cell cycle by serum-starvation (data not shown). Thus, the inability of cells expressing activated Ras to induce wt-p53 in response to serum deprivation is not due solely to their failure to become growth arrested, and conversely, the loss of wt-p53 inducibility in *Ki*-Balb cells may not be causally related to their failure to arrest cell cycle progression.

Dysregulation of p53-regulated genes *GADD45* and *MDM-2* in cells expressing activated *Ki*-Ras

The mammalian *GADD45* gene is highly conserved and can be induced by DNA damage and various other cellular stresses (Kastan *et al*, 1992). As a downstream effector of p53, the activity of the product of the *GADD45* gene appears to be involved in enhancement of DNA repair and inhibition of cellular DNA synthesis (Walker, 1984; Fornace *et al*, 1988, 1989; Kastan *et al*, 1992; Smith *et al*, 1994). Since the expression of p53 protein was reduced in *Ki*-Balb cells, the expression of *GADD45* gene was examined under normal growth conditions, or after serum-depletion, in Balb cells and *Ki*-Balb cells (Figure 3a). *GADD45* mRNA was detectable under normal growth conditions, and significantly induced (threefold) after serum-depletion in Balb cells. In contrast, *Ki*-Balb cells expressed no appreciable levels of *GADD45* under either normal growth or serum-depleted conditions. This dysregulation of *GADD45* expression therefore correlated with the observed disruption of p53 induction in these cells, and suggested that there might be a concurrent reduction of cellular damage repair capability in the presence of oncogenic *Ki*-Ras.

Deregulation of *MDM-2* gene expression has been demonstrated in various human tumors and transformed mouse cell lines. *MDM-2* transcription can be induced by wt-p53 (Cahilly-Snyder *et al*, 1987; Fakharzadeh *et al*, 1991). Conversely, the MDM-2 protein is a cellular inhibitor of p53, in that it can bind p53 and downregulate its activity (Momand *et al*, 1991; Chin *et al*, 1992; Prives, 1993). To determine if *MDM-2* expression was altered in the setting of oncogenic *Ki*Ras, analysis of *MDM-2* gene transcripts was conducted *Ki*-Balb cells (Figure 3b). *MDM-2* mRNA was

almost undetectable in Balb cells under normal growth conditions, but became abundant (6–8-fold induction) after serum-depletion. In contrast, appreciable amounts of *MDM-2* mRNA were expressed in *Ki*-Balb cells under normal growth conditions (1.8-fold higher than present in Balb cells), and there was no further induction of the *MDM-2* gene by serum-depletion, raising the possibility that this dysregulation of *MDM-2* gene induction may play a role in the disruption of p53 signaling observed in *Ki*-Balb cells, through disruption of the p53/MDM-2 autoregulatory feedback loop.

Enhancement of *CAD* gene amplification in cells expressing oncogenic *Ki*-Ras

Normal cells growth arrest in the presence of N-(phosphonacetyl)-L-aspartate (PALA), a specific inhibitor of *de novo* uridine biosynthesis. Cells lacking wt-p 53 form colonies in PALA at high frequency, presumably through a mechanism in which the *CAD* gene is amplified (Livingstone *et al*, 1992; Yin *et al*, 1992; Schaefer *et al*, 1993; Kelly *et al*, 1995). The *CAD* gene encodes a single polypeptide chain containing carbamyl phosphate synthetase, dihydroorotase and aspartate transcarbamylase (which PALA inhibits). Amplification of genes like *CAD* is facilitated under conditions of genetic instability (Windle *et al*, 1991; Stark *et al*, 1993). To investigate whether the loss of wt-p53 in *Ki*-Balb cells correlates with an increased frequency of *CAD* gene amplification in response to PALA treatment, the plating efficiency, sensitivity to PALA, and *CAD* gene amplification potential were tested and compared between the control Balb and *Ki*-Balb cells. After determination of the plating efficiency for each cell type, the PALA concentration that inhibited 50% of colony formation (LD_{50}) was defined, and the frequency of colonies arising which were resistant to six times this concentration was measured (Table 1). PALA-resistant clones were not detected in 1×10^7 Balb cells in the presence of PALA ($6\times LD_{50}$) (frequency of $<1/10^7$), but 17 PALA-resistant colonies were generated from 1×10^6 *Ki*-Balb cells (frequency of $>1/10^5$).

To verify that the *CAD* gene was amplified in the PALA-resistant clones, Southern blot analysis was conducted. Two resistant colonies of *Ki*-Balb cells were isolated after selection with PALA. DNA from the drug-resistant clones, as well as DNA from normal control Balb cells, after 10 days of PALA treatment was prepared. Each PALA-resistant clone of *Ki*-Balb cells demonstrated amplified copies of the *CAD* gene, whereas there was no *CAD* gene amplification in the control Balb cells (Figure 4), demonstrating that *CAD* gene amplification had occurred in the activated Ras-expressing cells.

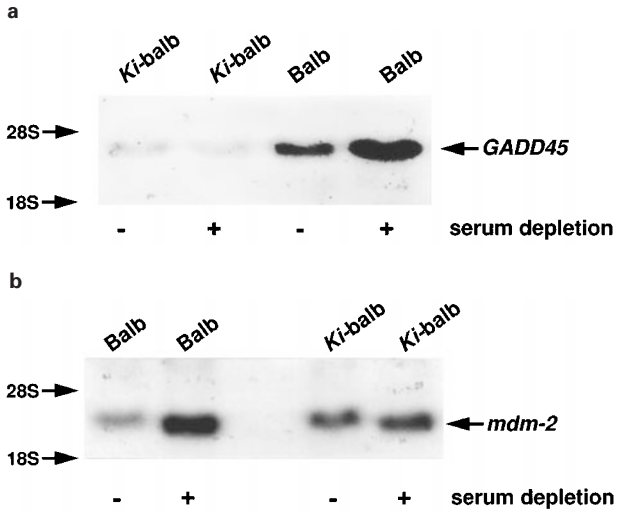


Figure 3 Induction of *GADD45* and *MDM-2* genes in the setting of serum-depletion. (**a**) Northern blot of *GADD45* mRNA message from Balb and *Ki*-Balb cells. Total RNA from the two cell types with (+) or without (−) serum-starvation were prepared, separated on an agarose gel, transferred to nitrocellulose, and probed with a ^{32}P-labeled hamster *GADD45* probe. (**b**). RNA blot of *MDM-2* gene expression. Total RNA from Balb and *Ki*-Balb cells with (+) or without (−) serum starvation were prepared, transferred to nitrocellulose, and the blot was hybridized with a ^{32}P-labeled murine *MDM-2* specific probe. Equal loading of total RNA into each lane of the gel for each experiment was ensured by rehybridization of the blot with a *β-actin* probe (data not shown)

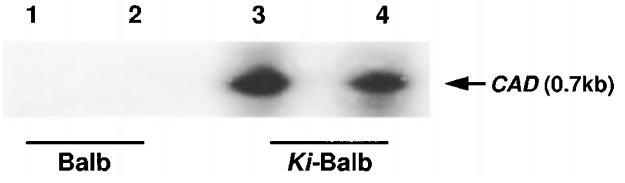


Figure 4 *CAD* gene amplification in the presence of PALA. DNAs were isolated from Balb cells (lanes 1 and 2), and two PALA-resistant *Ki*-Balb clones (lanes 3 and 4) after selection in 30 μM of PALA for Balb cells and 90 μM of PALA for *Ki*-Balb cells. DNAs were normalized after restriction digestion, separated on an agarose gel, and hybridized with a 0.3 kb mouse *CAD* cDNA probe, a product of PCR

Table 1 Gene amplification in Balb and *Ki*-Balb mouse fibroblasts

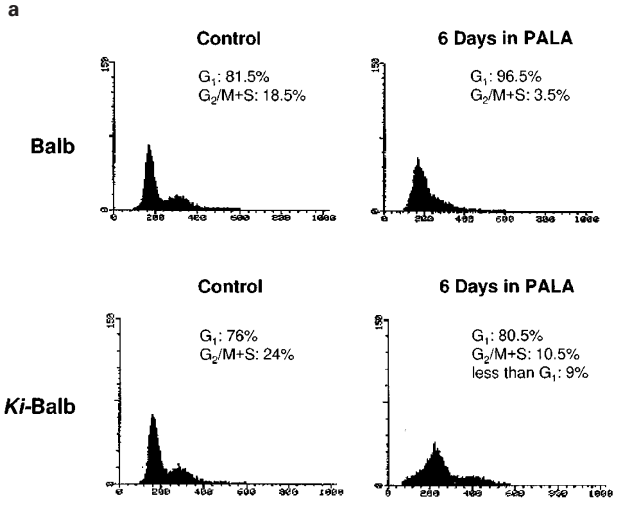
Cell	PE (%)	p53	2 Days Serum-depletion	PALA LD_{50} (μM)	PALA Colonies	CAD gene Amplification Frequency at $6\times LD_{50}$
Balb	17.5	+	−	5 μM	$0/1\times10^7$	ND ($<1\times10^7$)
Balb	17.5	+	+	5 μM	$0/1\times10^7$	ND ($<1\times10^7$)
Ki-Balb	25	−	−	12 μM	$17/1\times10^6$	$1\times10^5\pm4\times10^4$
Ki-Balb	25	−	+	12 μM	$0/1\times10^7$	

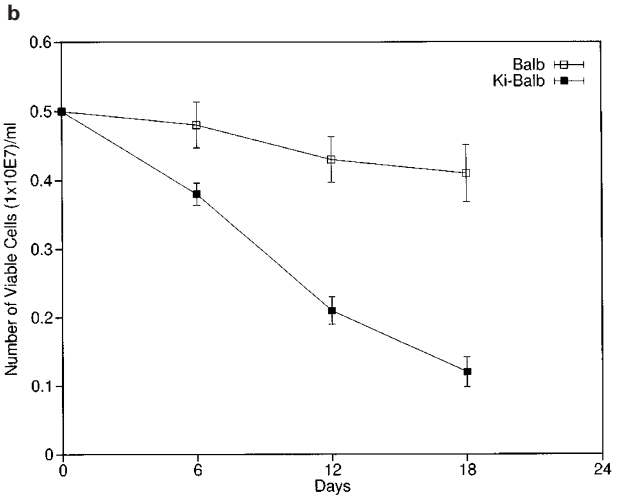
The frequencies of PALA resistance were determined as described in the Methods and Materials. PE represents plating efficiency. p53 refers to the expression of wt-p53 protein. LD_{50} is the concentration of PALA that reduces colony forming ability by 50%. ND indicates that no PALA-resistant colonies were detected

PALA-induced apoptosis accompanied by loss of PALA-mediated G1 arrest in cells expressing oncogenic *Ki*-Ras

Since *Ki*-Balb cells cannot be arrested in cell cycle by serum-depletion, the effect of PALA on cell cycle progression was determined (Figure 5a). The DNA profiles of the cells were analyzed at day 6 of PALA treatment, and compared with profiles from the same cells cultured under normal growth conditions. The majority of Balb cells accumulated in the G1-phase in the presence of PALA. In contrast, *Ki*-Balb cells remained distributed throughout the cell cycle, indicating that oncogenic Ras perturbed PALA-induced cell cycle arrest.

The effect of PALA at higher doses on cell viability also differed between these two cell types (Figure 5b). Cell viability in the presence of PALA at $8 \times LD_{50}$ was assayed over a 3 week period and was quantitated at 6-day intervals by trypan blue exclusion. More than 80% of Balb cells were still viable at the end of 3 weeks, and most of the viable cells remained arrested in G1-phase during this period. In contrast, far fewer *Ki*-Balb cells survived the $8 \times LD_{50}$ concentration of PALA, but at the same time, those cells which did survive were able to form colonies in the presence of PALA, demonstrating that they remained in cell cycle. To determine whether the decrease in survival of *Ki*-Balb cells during PALA treatment was due to apoptosis, the percentage of the cells with fragmented DNA, a characteristic feature of apoptosis, was measured by staining a portion of the same cells shown in Figure 5b with propidium iodide at 6-day intervals, followed by nuclear DNA analysis on a fluorescence-activated flow cytometer (Figure 5c). The percentage of *Ki*-Balb cells with fragmented DNA increased, and persisted, at a level of more than 10% for the 18-day treatment period, while in control Balb cells no significant increase in DNA fragmentation was observed. This suggested that loss of the viability of *Ki*-Balb cells during PALA selection was due to apoptosis. Taken together with the data from Figure 5a, it also suggested that this PALA-mediated apoptosis in the





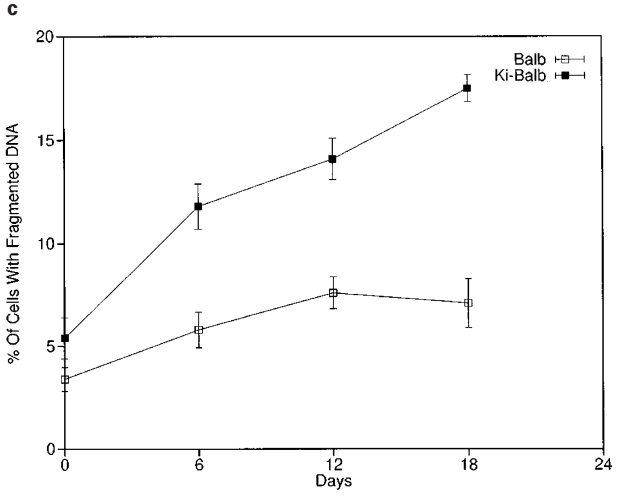


Figure 5 Loss of growth arrest and of viability mediated by PALA in *Ki*-Ras-expressing cells. (**a**) Flow cytometric DNA profiles of Balb and *Ki*-Balb cells after 6 days of PALA selection, as compared to cells grown without PALA selection. The percentages of cells in G1, S, and G2/M are indicated. (**b**) Five replicate plates of Balb and *Ki*-Balb cells were cultured in a concentration of $8 \times LD_{50}$ PALA (40 μM for Balb cells and 120 μM for *Ki*-Balb cells), and viable cells were enumerated at 6-day intervals for 18 days. (**c**) Nuclear DNA fragmentation in the same cell lines in the presence of PALA used at the concentrations described in **b**, was analyzed at 6-day intervals for 18 days

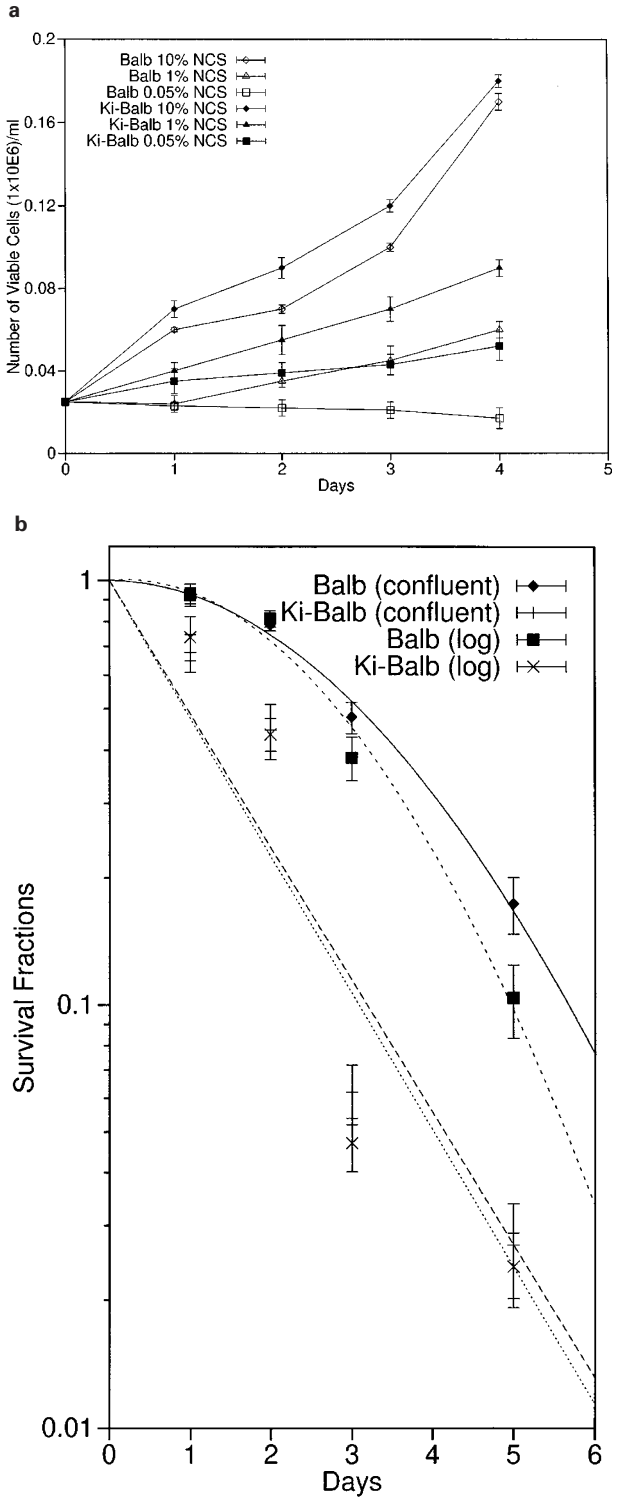


Figure 6 Cell kinetics with or without serum withdrawal and hypothetical extrapolated survival curves for Balb and *Ki*-Balb cells after various times of serum-starvation in a clonogenic assay. (**a**) Five replicate wells of Balb or *Ki*-Balb cells were cultured in medium containing various concentrations of newborn calf serum as indicated. Viable cells were enumerated at daily intervals for 4 consecutive days. Error bars indicate standard errors. (**b**) Balb and *Ki*-Balb cells cultured under log growth or confluent conditions were subjected to different time periods of serum-starvation. Afterwards, clonogenic assays were performed. The fraction of surviving cells is plotted on a logarithmic scale against duration of serum-starvation on a linear scale. The coefficients α and β relate to the initial slope and final slope, respectively

presence of oncogenic Ras, like the formation of PALA-resistant colonies, may be dependent upon Ras-induced transit through the cell cycle beyond G1. However, this program of cell death was simultaneously accompanied by the generation of PALA-resistant colonies, which indicates that the genetic instability caused by oncogenic Ras may simultaneously contribute to two opposing biological processes: gene amplification conferring resistance to environmental stresses and enhanced sensitivity to apoptosis.

Reduction of clonogenicity in cells expressing oncogenic *Ki*-Ras following serum-depletion

Constitutive expression of *myc* results in rapid apoptosis after withdrawal of serum (Evan *et al*, 1992). To explore the effects of activated Ras under the same conditions, control Balb cells and *Ki*-Balb cells were cultured in medium containing various concentrations of newborn calf serum and the viable cells were enumerated at daily intervals over 4 consecutive days (Figure 6a). The growth rate of Balb cells was reduced with decreasing concentrations of serum. The number of Balb cells remained approximately the same (no growth) in medium containing 0.05% serum over the 4-day interval. The cell growth kinetics of *Ki*-Balb cells were moderately elevated in medium containing 10% serum in comparison with Balb cells. At the lower concentrations of serum, the growth rate of *Ki*-Balb cells was relatively decreased, but the cells continued to proliferate, even at 0.05% serum. This confirmed previous observations that constitutively-activated Ras, to some extent, may still provide signals for cell cycle progression and production of viable daughter cells under conditions of serum-starvation. Cell viability was also determined by DNA fragmentation analysis (data not shown). There was no obvious increase in the percentage of cells with fragmented DNA in either Balb or *Ki*-Balb cells over the four days of serum-starvation.

Since the cells expressing oncogenic *Ki*-Ras did not die during the 4-day period of serum-starvation, we next assessed whether there was an impact of prolonged serum-starvation on the longevity/propagation capability, or on the repair ability, of these cells with their propensity for genomic instability due to the presence of activated Ras. Colony formation assays were conducted following various periods of incubation in medium depleted of serum. Balb cells and *Ki*-Balb cells, initially under either confluent or log growth conditions, were cultured in medium containing 0.05% of serum for various time intervals as indicated, and the viable cells were subsequently replated for clonogenicity assays. For each experiment, parallel control dishes were set up for determination of the plating efficiency (PE). The empirical survival curve model (α/β model), which has been widely used in radiobiology to access cell sensitivity to radiation or the capacity to repair radiation-induced damage (Chen and Geard, 1990; Fisher,

1994; Hallberg *et al*, 1994; Hall, 1994), was employed to analyze the results of this assay (Figure 6b). α represents the linear component of the slope and β is the final nonlinear component of the slope of the cell survival curve in response to various inducers of cellular damage. The survival curves for Balb and *Ki*-Balb cells are presented as duration of serum-depletion plotted on a linear scale with surviving fraction on a logarithmic scale. The survival curves of *Ki*-Balb cells under both conditions (whether initially confluent or in 'log phase'), were approximately linear with similar α components. The lines for the Balb cells were nonlinear under both confluent and 'log' conditions. In analyzing such plots in the assessment of survival after radiation exposure, a non-linear survival curve, like that seen for the Balb cells, suggests the need for multiple 'hits' to induce cell death; in contrast, a linear curve, as seen for the *Ki*-Balb cells, implies the need for only a single 'hit'. One interpretation of these results could be that the presence of activated Ras might provide the first hit, either through accumulation of genetic damage or disruption of repair capability. The latter part of the slope of the curve (the β component) from 'log phase' Balb cells became steeper than the β component from the confluent Balb cells, which may reflect that these normal cells may have differing sensitivities to persistent serum-starvation in different phases of the cell cycle. Under confluent conditions, the majority of Balb cells resided in the G0/G1-phase of the cell cycle as a result of contact inhibition. Cells in G1-phase may be less sensitive to, or more efficient in repair of, the damage or stress caused by extended withdrawal of growth factors. Since transformed *Ki*-Balb cells lose contact inhibition as well as their G1 cell cycle checkpoint, the effect of prior confluence or log growth conditions may not alter the intrinsic sensitivity of these cells to serum-depletion.

Loss of PALA-resistant colony formation potential in the presence of activated *Ki*-Ras following serum-starvation

The clonogenicity experiments detailed above suggested that the loss of longevity or propagation ability of *Ki*-Balb cells following persistent serum-starvation may be due to the accumulation of genetic errors or the inability to repair cellular damage. To determine if *Ki*-Balb cells retained the ability to generate PALA-resistant colonies following serum-starvation, Balb and *Ki*-Balb cells were incubated in medium with 0.05% of newborn calf serum for 2 days, and then cultured in normal growth medium in the presence of PALA for colony-forming assays. The 2 day period of serum-starvation was determined from Figure 6, which showed that the majority of the cells were viable after this interval of serum-starvation, and that 30 – 40% of seeded *Ki*-Balb cells would still form colonies. No PALA-resistant colonies arose from 1×10^8 plated Balb cells after 2 days of prior serum starvation (Table 1). *Ki*-Balb cells also failed to generate any PALA-resistant colonies when seeded in the same numbers as the Balb cells.

The effect of PALA on induction of apoptosis were examined using this combination of serum-starvation and PALA treatment. Cells were cultured in medium containing 0.05% serum for 2 days, and then returned to normal growth conditions in the presence of PALA at $8 \times LD_{50}$ for 6 days. Subsequently, cellular DNA fragmentation analysis was conducted (Figure 7). The percentage Balb cells exhibiting DNA fragmentation after the combination treatment was slightly, but not significantly, higher than in Balb cells growing under normal conditions (4.2 *vs*. 3.5%). However, the majority of the Balb cells remained viable and in a state of growth arrest (data not shown). Prior serum-starvation treatment increased the percentage of PALA-mediated DNA fragmentation in *Ki*-Balb cells (to about 18.5% at day 6) in comparison with the cells treated with PALA without prior serum-starvation (about 11%). Overall, these results imply that the *CAD* gene amplification induced by (or permitted by) the presence of the *Ki-ras* oncogene was suppressed by prior serum-starvation of the cells.

Discussion

The development and progression of cancer is a multistep process. Molecular and cytogenetic examinations have revealed that the progressive stages of tumorigenesis are driven by stochastic accumulation of genetic and epigenetic changes in tumor cells. Loss of the function of certain tumor suppressors can eliminate a cell cycle checkpoint (G1/S) and enhance the frequency of genetic errors through genomic instability. The studies presented here demonstrate that the susceptibility to restructuring of the transformed cell genome by oncogenic Ras, through the disruption of the expression of tumor suppressor p53, not only confers some activities predisposing to cancer cell development, but also may render the same cells more susceptible to apoptotic processes. We also demonstrate that such cells with such unstable genomes have an increased tendency to accumulate lethal genetic errors, or may be unable to repair cellular

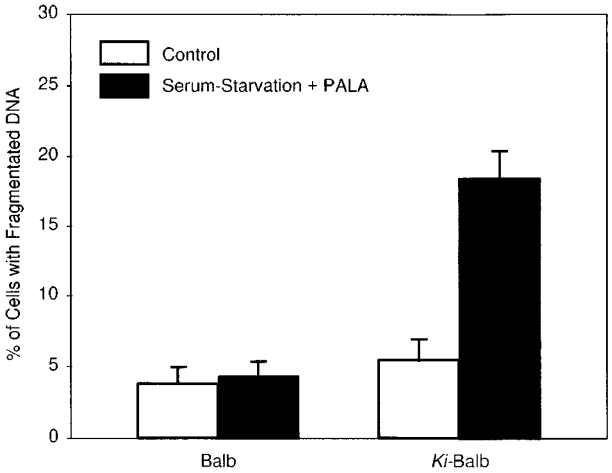


Figure 7 PALA-mediated DNA fragmentation following serum-starvation. The cells were cultured in the medium containing 0.05% of serum for 2 days, and subsequently cultured under normal growth conditions in the presence of $8 \times LD_{50}$ of PALA for 6 days. The DNA fragmentation of Balb and *Ki*-Balb cells were examined by flow cytometry. Error bars represent the standard error over five independent experiments

damage, when they are forced to enter the cell cycle aberrantly or prematurely. Both immediate apoptosis and loss of longevity or propagation ability are the results of this process.

The differential ability of Balb cells and *Ki*-Balb cells to form colonies in response to persistent serum-deprivation or to PALA-exposure raised the possibility that activated Ras may utilize a common bi-functional cellular component in directing the cells to undergo apoptosis or tumorigenesis. The tumor suppressor p53 has pleiotropic functions, including control of genomic plasticity and integrity, as well as modulation of repair pathways. Cells with impaired p53 have been shown to be genetically unstable, owing to loss of cell cycle checkpoint control and accumulation of misrepair events (Fornace *et al*, 1989; Livingstone *et al*, 1992; Yin *et al*, 1992; Carder *et al*, 1993; Schaefer *et al*, 1993; Lee *et al*, 1994; Bouffler *et al*, 1995; Donehower *et al*, 1995). Analysis of wt-p53 protein expression in the presence of oncogenic Ras showed that such cells have reduced levels of this tumor-suppressor protein in both nuclear-free cell lysate and nuclear fractions. This disruption of the expression of wt-p 53 protein by activated Ras was negatively-correlated with the capacity for PALA-mediated cell cycle arrest, and positively-correlated with an increased frequency of PALA-selected *CAD* gene amplification and an increased susceptibility to PALA-induced apoptosis. Mutation or inactivation in genes with diverse functions such as *p53* may contribute to abnormal chromosomal rearrangements in cells, including gene amplification. Genes encoding protein that participate in DNA replication or repair can also confer genomic instability when mutated. Amplified gene structures may be formed in both normal and tumor-prone cells, but can be repaired in normal cells (Tlsty, *et al*, 1989; Tlsty, 1990). For instance, transfection of wt-p53 into the fibroblasts from Li-Fraumeni syndrome patients lacking wt-p53 expression will restore G1 cell cycle arrest and inhibit *CAD* gene amplification (Livingstone *et al*, 1992; Yin *et al*, 1992). The data presented herein demonstrate that in cells expressing oncogenic Ras, exposure to PALA efficiently induced programmed cell death, and this PALA-mediated susceptibility to apoptosis was also associated with the capacity to generate PALA-resistant colonies. One possible interpretation of this observed correlation between genetic instability and susceptibility of apoptosis induced by PALA in the presence of activated Ras could be that the loss of the PALA-mediated gating mechanism at the G1/S checkpoint, perhaps together with a defect in proof-reading mechanisms, may initiate replication-associated chromosome breakage and the generation of acentric fragments containing target genes, leading to a gene amplification process in a small proportion of the *Ki*-Balb cells. For the majority of the *Ki*-Balb cell population, however, the increased apoptotic activity of PALA mediated by oncogenic Ras may be secondary to its known ability to reduce the intracellular pools of nucleotides, such as dTTP, dCTP, dGTP and UTP (Moyer *et al*, 1982). Premature transition through S-phase under limited concentrations of dTTP alone is sufficient to cause apoptosis (Koyama *et al*, 1982; Ayusawa *et al*, 1983).

Activated Ras does not promote the apoptotic process during short-term serum-starvation (Chen and Faller, 1995). Here, our data further demonstrated that during 4 days of serum depletion, oncogenic *Ki*-Ras continues to act as a mitogenic signal transducer to drive *Ki*-Balb cells to proliferate, although the cell doubling time was modestly extended in the absence of serum. However, the colony formation assay showed that the ability of *Ki*-Balb cells to generate colonies was dramatically reduced with persistent serum-starvation. Interestingly, the survival curves of Balb and *Ki*-Balb cells in response to different time periods of serum-depletion fit the prominent model describing cellular sensitivity to radiation, in which an increasing slope of the curve correlates with enhanced radio-sensitivity of cells perhaps through lack of ability to repair DNA breaks caused by radiation. Therefore, without proper cell cycle checkpoint surveillance as part of genetic control, cell cycle progression under the condition of persistent serum-starvation may cause cellular damage or stress, and cells deficient in repair processes would be sensitive to this stress, leading to loss of longevity or propagation ability or apoptosis. The discrepancy found between the assays of the cell growth kinetics during serum-starvation and the colony formation after serum-depletion in *Ki*-Balb cells may thus reflect two different functions of oncogenic *ras* in two different 'environments'. During conditions of low-serum concentration, the mitogenic function of oncogenic Ras may compensate for certain growth factors in the serum to promote cell proliferation within certain time periods; but at the same time, however, without the control conferred by the G1 checkpoint and with impaired repair machinery (such as *GADD45*-involved repair processes), oncogenic Ras may accelerate the accumulation of lethal genetic errors which may affect the longevity of the cells. This cumulative genetic damage may then be revealed by the clonogenicity assay. In a clonogenicity assay after persistent serum-depletion, cells must be able to repair or recover from the preceding damage or stress to form colonies. In the presence of oncogenic Ras, however, such repair machinery may become inefficient or inaccurate with increasing time periods of serum-starvation.

Both serum-starvation and PALA-exposure can arrest normal Balb cells in G1 of the cell cycle, but Balb cells expressing activated Ras do not arrest under either condition. The differential ability of *Ki*-Balb cells to form colonies following these two stresses may derive from a common, unstable genetic background, but may be due to two different mechanisms. Inability of *Ki*-Balb cells to propagate following persistent serum-starvation may indicate a defect in the processes of repair of cellular damage. Yet, in the presence of PALA selection, a small fraction of the *Ki*-Balb cells are able to form PALA-resistant colonies because they were permissive for *CAD* gene amplification, indicating impaired recombination machinery or processes during DNA replication. The combination of serum-starvation plus PALA-exposure in cells expressing oncogenic Ras, however, appeared to synergize the apoptotic process. The cell cycle transit through the G1 checkpoint enforced by oncogenic Ras in the absence of certain growth factors (serum) may inhibit or damage the

genes which may subsequently regulate the initiation of the breakage-fusion-bridge cycle triggered by PALA to facilitate the *CAD* gene amplification and the formation of PALA-resistant colonies. However, it is also reasonable to postulate that oncogenic Ras may interfere with tumor suppressors, such as p53, and further lead cells to undergo numerous genomic alterations by affecting different genetic mechanisms. A genome so changed has the potential not only for tumorigenesis but also for increased susceptibility to cell death, depending upon the circumstances.

The functions of oncogenic Ras, as a pro- or anti-proliferative effector, are the subject of some controversy. Oncogenic Ras has been demonstrated to cause proliferative arrest and differentiation in two rodent cell lines, rat pheochromocytoma PC12 cells and rat Schwann cells (Ridley *et al*, 1988; Bar-Sagi and Feramisco, 1985). A recent report has documented that expression of oncogenic *H-rasV12* in either primary human diploid fibroblasts (IMR90) or primary mouse embryo fibroblasts (MEFs) results in G1 arrest, and such cell progression arrest is accompanied by accumulation of p53 and p16 (Serrano *et al*, 1997). Conversely, inactivation of tumor suppressors are the most prevalent mutations in some human tumors accompanying oncogenic mutations of the *ras* gene (Hick *et al*, 1991; Lu *et al*, 1992; Tanaka *et al*, 1994; Weinberg, 1995; Serrano *et al*, 1996; Hollstein *et al*, 1996). This discrepancy of oncogenic Ras actions on regulation of G1 arrest of cell cycle may be cell-type specific, or due to particular steps in tumorigenic development, or to ectopic expression of the oncogene and its subsequent functions, or even to the duration of expression of oncogenic Ras. The cells employed in this investigation are early passage mouse Balb/c-3T3 fibroblasts. These cells still possess many properties of primary cells, such as contact inhibition of cell division, growth at a high dilution, and lack of *CAD* gene amplification, but are more sensitive to transformation than many primary cell cultures. It is possible that, in the course of establishing Balb cells in culture, some, as yet unknown, intrinsic mutations have occurred which may co-operate with oncogenes, including *ras*, to disrupt the G1/S cell cycle checkpoint and allow full transformation. It is noteworthy, however, that we have observed similar effects of oncogenic Ras when it is introduced into a human T lymphoblastoid line or into primary human endothelial cells (unpublished).

The present findings may begin to establish connections among disruption of p53, gene amplification, tumorigenesis and susceptibility to apoptosis in the presence of oncogenic Ras. These results are consistent with a model encompassing the dual functions of growth-related genes, such as *myc* and *ras* (Evan *et al*, 1992; Harrington, et al. 1994; Gulbins *et al*, 1995). Independent of the transformation process, we have shown that cell expressing oncogenic or transiently but inappropriately-activated Ras become more vulnerable to certain environmental changes, such as modulation of PKC activity (Chen and Faller, 1995; Chen, *et al*, 1998). Under such conditions, the progression of the cells towards two distinct and opposite biological processes, proliferation and apoptosis, is determined by both intrinsic biological or genetic status (e.g. alterations in tumor suppressors) and exogenous inputs (e.g. modulation of enzyme levels). These findings are consistent with a model encompassing the dual functions of proto-oncogene products such as Myc and Ras. Signals for proliferation in normal cells, mediated in part by Ras and Myc, may actively and appropriately suppress the apoptotic process. Under certain conditions, Ras and Myc probably also activate apoptotic pathways to provide a control against unlimited growth. Thus, the same cellular machinery may operate or regulate these two linked processes of cell proliferation and apoptosis. In oncogenic transformation, the signals for proliferation are enhanced by proto-oncogenes, and the conflict between cell growth and apoptosis may be also intensified. The ability to induce a clash of these two processes by manipulation of the cellular environment may provide a new and promising approach to oncogene-directed cancer therapy.

Materials and Methods

Cell culture and cell lines

Mouse fibroblasts, Balb/c-3T3, clone A31 initially developed from 14-day-old Balb/c mouse embryos, were obtained from the American Type Culture Collection (Rockville, MD, USA). The Balb cells were cultured for one or two passages after purchase and aliquoted into freezing vials. For each experiment, freshly-thawed cells were used, which possessed primary cell-like, non-tumorigenic and contact-inhibited characteristics. Retroviral vector stocks containing *v-Ki-ras* or c-*myc* cDNAs were derived from cells transfected with cloned vectors as previously described (Zullo and Faller, 1988). After infection, transfectants were assayed for constitutive expression of *ras* or *myc* mRNAs by Northern blot analysis and proteins by immunoblot analysis. Subsequently, cells were maintained in Dulbecco Modified Eagle medium (DME) supplemented with 10% heat-inactivated newborn calf serum (Hazelton Research Products, Inc., Lenexa, KA, USA), 2 mM L-glutamine, 100 U penicillin per ml, 100 μg streptomycin per ml, and geneticin or hygromycin selection where appropriate. For cell growth kinetic assays, cells (at 0.25×10^6 cells/ml) were incubated in medium containing different percentages of newborn calf serum, and the number of viable cells were determined daily by trypan blue exclusion.

Cell viability assay

Cells were cultured in medium containing different concentrations of newborn calf serum or PALA for the time periods as indicated in the figures. Viable cells were enumerated at daily intervals using trypan blue exclusion.

DNA content and cell cycle distribution analysis

After culture in medium containing 10 or 0.05% of newborn calf serum for 48 h, cells (1×10^6) were washed with $1 \times$ PBS twice, fixed with 70% ethanol, and subsequently resuspended in 1 ml of 1% Na citrate, 0.1% Triton X-100 and 50 μg/ml of propidium iodide. DNA profile analysis was performed with a FACScan (Becton Dickenson, Mountain View, CA, USA). The data analysis and display were performed using the Cell-Fit software program (Becton Dickenson, Mountain View, CA, USA). Cell-Fit provides data from the flow cytometer for real-time

statistical analysis, computed at 1 s intervals, and also discriminates doublets or adjacent particles. The same procedure of the DNA profile analysis was conducted for the PALA selection procedures, wherein the cells were grown in medium with or without PALA at $8 \times LD_{50}$ for 6 days, prior to the analysis.

Cell survival assay

A cell survival assay was used to examine the ability of single cell to form colonies after various periods of serum-starvation. After culturing cells in medium containing either 10 or 0.05% newborn calf serum for various time periods as indicated, Balb and *Ki*-Balb cells were trypsinized, and re-plated in 100 mm dishes at suitable dilutions. The incubations were carried out at 37°C for 14 days, after which the colonies obtained were fixed, stained, and counted. The plating efficiency for each cell line was established by determining the number of cells required to yield 50 colonies per dish under normal growth conditions.

Immunoprecipitation and Western blot analysis

After growth in the medium containing 10 or 0.05% newborn calf serum for 48 h, cells (20×10^6), were trypsinized and washed with $1 \times$ PBS three times. The cells were resuspended in lysis buffer containing 20 mM Tris-HCl (p H 7.5), 2 mM EDTA, 5 mM EGTA [ethylene glycol-bis (ζ-aminoethyl ether)-N,N,N′,N′-tetraacetic acid], 10 mM β-mercaptoethanol, and 10 ηg of leupeptin and aprotinin per ml, and passed through a 22-gauge needle several times. The lysates were centrifuged at $280 \times g$ for 10 min at 4°C. The supernatants were collected as nuclear-free cell lysates and the pellets as nuclei. Subsequently, nuclei were resuspended in 50 μl of nuclear buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride. The protein concentrations in the nuclear-free cell lysates or the nuclear extracts were normalized. Equal amounts of total protein from different cell fractions were immunoprecipitated by using anti-mouse-p53 antibody (Oncogene Science, Uniondale, NY, USA), and subsequently immunoblotted with the same antibody. The immunoblots were developed with an anti-mouse Ig alkaline phosphatase reagent (Oncogene Science).

RNA blot analysis

Total cellular RNA was isolated by guanidine thiocyanate/phenol RNA extraction, quantified, separated by electrophoresis on formaldehyde-agarose gels, and transferred to nitrocellulose (Costar, Cambridge, MA, USA). Hybridization and high stringency wash were performed as previously described (Faller *et al*, 1994). [^{32}P]-labeled DNA probes were made by the random oligonucleotide primer method (Feinberg and Volgelstein, 1983). The *GADD45* probe was a 1.2 kb *Kpn*I to *Sac*I fragment of hamster *GADD45* (generously provided by Dr. A. Fornace). The *MDM-2* probe was a 1.3 kb *Eco*RI fragment of murine *MDM-2* (generously provided by Dr. A. Fornace).

PALA selection

Balb and *Ki*-Balb cells were grown in the presence of various concentrations of PALA, a drug that specifically inhibits the aspartate transcarbamylase activity of the multifunctional CAD enzyme and selects for cells with amplified copies of *CAD* gene (obtained from the Drug Synthesis Branch, Division of Cancer Treatment, National Cancer Institute). Subsequently, the concentration of PALA that inhibited colony-forming ability by 50% (LD_{50}) was determined for each cell line. Cells were selected to resist to PALA (at $6 \times LD_{50}$) and be able to grow to form colonies in 4–5 weeks. Frequency of *CAD* gene amplification was expressed as the number of colonies formed in $6 \times LD_{50}$ of PALA relative to the number of colonies formed without PALA.

Southern blot analysis

DNA was prepared from the colonies of *Ki*-Balb cells after PALA selection as described above as well as from normal Balb cells. The genomic DNA was digested with restriction endonucleases, precipitated with EtOH, and subsequently dissolved in TE. Equal amounts of digested genomic DNA was loaded in each lane of the agarose gel, fractionated by electrophoresis, and transferred to nitrocellulose. The mouse *CAD* probe was generated by PCR as a 300 bp of DNA fragment using primers (5′-GAGCGAGATACACG-CACGTTGCA and 3′-CCTGAATGAACTTGGAGATGACTA), radio-labeled by the random primer method, and hybridized to the nitrocellulose filters.

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