The EMBO Journal vol.14 no.3 pp.461–472, 1995

Inhibition of apoptosis by the retinoblastoma gene product

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Communicated by B.Westermark

Tissue homeostasis and the prevention of neoplasia require regulatory co-ordination between cellular proliferation and apoptosis. Several cellular proteins, including c-myc and E2F, as well as viral proteins such as E1A, have dual functions as positive regulators of apoptosis and proliferation. The product of the retinoblastoma tumor suppressor gene, pRb, binds these proteins and is known to function in growth suppression. To examine whether pRb may function as a negative regulator of both proliferation and apoptosis, we analyzed apoptosis induced in transfected derivatives of the human osteosarcoma cell line SAOS-2. Ionizing radiation induced apoptosis in a time- and dose-dependent manner in SAOS-2 cells, which lack pRb expression. In both a transient and stable transfection assay, SAOS-2 derivatives expressing wild-type (wt) pRb exhibited increased viability and decreased apoptosis following treatment at a variety of radiation doses. Expression in SAOS-2 of a mutant pRb that fails to complex with several known binding partners of pRb, including E1A and E2F, did not protect SAOS-2 cells from apoptosis. Radiation exposure induced a G_2 arrest in SAOS-2 and in derivatives expressing pRb. Inhibition of DNA synthesis and cell cycle progression by aphidicolin treatment failed to protect SAOS-2 cells or pRb-expressing isolates from undergoing apoptosis. Our data document a novel function for pRb in suppressing apoptosis and suggest that several proteins shown to induce apoptosis, including E1A, E2F and c-myc, may do so by interfering with the protective function of pRb.

Key words: apoptosis/DNA damage/ionizing radiation/RB

Introduction

Apoptosis is a process of programmed cell death that plays an important role in physiological processes such as immune- and nervous-system development (Cohen *et al.*, 1992; Golstein *et al.*, 1991; Oppenheim, 1991; Rothenberg, 1992), and contributes to defense mechanisms important for the prevention of infectious illness and cancer (Clem *et al.*, 1991; Hershberger *et al.*, 1992; Rao *et al.*, 1992). Hallmarks of apoptosis include decreased cell size, shrinking cytoplasmic volume, chromatin fragmentation and sustained plasma membrane integrity (Dive *et al.*, 1992). Apoptosis is a genetically controlled process, and genes important for the regulation of apoptosis have recently been identified (Vaux, 1993). These include p53 (Lowe *et al.*, 1993b), *myc* (Evan *et al.*, 1992) and other genes (Williams and Smith, 1993) first recognized to play a role in cell growth and differentiation.

Apoptosis may sustain tissue homeostasis by balancing the effects of proliferation. The regulatory coupling of proliferation and apoptosis is suggested by several recent findings. A line of investigation relevant to this hypothesis has focused on features shared by the pathways of apoptosis and proliferation. These studies have revealed that entry into S phase, an initial step in cell cycle progression, may also occur in the pathway of cell death (Colombel *et al.*, 1992). One such study of prostate epithelial cells showed that apoptosis induced by testosterone withdrawal is preceded by re-entry into the cell cycle: after receiving a stimulus that induces apoptosis, quiescent prostate epithelial cells enter S phase and then die without completing the cell cycle. A second line of investigation has focused on regulatory genes that control both proliferation and apoptosis (Lee *et al.*, 1993). Expression of genes important for cell cycle regulation, including cyclin D, myc, E1A and E7, is associated not only with proliferation, but also with cell death (Evan *et al.*, 1992; Rao *et al.*, 1992; Freeman *et al.*, 1994; Pan and Griep, 1994; White *et al.*, 1994).

Cyclin D1 expression is required for serum-stimulated fibroblasts to progress through G_1 and enter S phase (Baldin *et al.*, 1993; Quelle *et al.*, 1993). Conversely, overexpression of cyclin D1 forces rapid transit through G_1 and decreased cell cycle duration (Quelle *et al.*, 1993). Recently, a specific induction of cyclin D1 expression was demonstrated in neurons induced to undergo apoptosis following nerve growth factor withdrawal (Freeman *et al.*, 1994). Expression of the proto-oncogene c-myc is also associated with coupled proliferative and apoptotic functions. C-myc is a positive growth regulator and signals entry into the cell cycle (Eilers *et al.*, 1991; Shibuya *et al.*, 1992). In the setting of serum withdrawal, however, constitutive c-myc expression causes apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992). A dual function in proliferation and apoptosis is exhibited not only by cellular proteins, but also by viral gene products. The viral oncoproteins E7 and E1A, of the human papilloma virus and adenovirus, respectively, mediate both proliferation

and cell death (Rao *et al.*, 1992; Pan and Griep, 1994; White *et al.*, 1994).

Cyclin D, c-myc, E7 and E1A share an important additional feature. All four proteins bind the retinoblastoma tumor suppressor protein and antagonize its function (Whyte *et al.*, 1988; Dyson *et al.*, 1989, 1992; Munger *et al.*, 1989; Rustgi *et al.*, 1991; Goodrich and Lee, 1992; Dowdy *et al.*, 1993). The retinoblastoma susceptibility protein functions as a negative growth regulator. The retinoblastoma tumor suppressor gene (pRb) binds the transcription factor E2F and prevents transcription of E2F-responsive genes such as dihyrofolate reductase and thymidine kinase, which are expressed in G_1 as the cell re-enters the cell cycle (Blake and Azizkhan, 1989; Chellappan *et al.*, 1991; Dou *et al.*, 1991; Helin *et al.*, 1992; Kaelin *et al.*, 1992). Additional cell cycle regulators known to bind pRb, such as Id-2 (Iavarone *et al.*, 1994) and cyclin D (Dowdy *et al.*, 1993; Ewen *et al.*, 1993), form a complex specifically with the active, hypophosphorylated form of pRb that is present in G_1. Phosphorylation of pRb at the G_1/S transition signals passage of the cell into S phase (Buchkovich *et al.*, 1989; Goodrich *et al.*, 1991).

Binding to pRb mediates the activities of viral oncoproteins as well as the functions of cellular regulators. The oncoproteins T antigen of SV40 and E1A and E7 of adenovirus and human papilloma virus, respectively, induce cellular proliferation by binding and inactivating the hypophosphorylated form of pRb (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Munger *et al.*, 1989). These transforming proteins complex with pRb via the same sequences that are required for their oncogenic activity (Whyte *et al.*, 1988; Nevins, 1992). The E1A sequences that are required for pRb binding and transformation are also responsible for E1A-mediated apoptosis. Therefore, the induction of apoptosis by E1A, and other oncogenic pRb-binding proteins, may result from pRb inactivation. We examined the hypothesis that pRb may inhibit not only proliferation and transformation, but also apoptosis. Such a possibility is consistent with reports of abnormally extensive apoptosis in *RB*-deficient mice (Clarke *et al.*, 1992; Morgenbesser *et al.*, 1994).

In this study, we focus on radiation-induced cell death to evaluate the role of pRb in suppressing apoptosis. Ionizing radiation serves as an excellent tool due to its documented ability to induce apoptosis (Clarke *et al.*, 1993; Lowe *et al.*, 1993b), its well-characterized effects on cell cycle progression (Hall, 1988) and its clinical relevance as a widely used cancer therapy. Our experiments indicate that pRb protects SAOS-2 cells, which lack both pRb and wild-type (wt) p53 expression (Huang *et al.*, 1988, and data not shown), from radiation-induced apoptosis. This novel function of pRb has important implications for both normal development and pathological states such as neoplasia.

Results

pRb expression is associated with increased radiation resistance

The characterization of cellular radiation resistance has been the subject of intense research for decades, not only because it may have clinical implications, but also because it reflects a culmination of numerous molecular pathways, including DNA repair, cell cycle checkpoint fidelity, oncogene expression and tumor suppressor function (Painter and Young, 1980; Denekamp, 1986; Weinert and Hartwell, 1988; Hartwell and Weinert, 1989; Kastan *et al.*, 1991, 1992; Weichselbaum, 1991; Kuerbitz *et al.*, 1992; Price, 1993). *In vitro* radiation resistance is generally measured by plotting the fraction of cells able to proliferate and form colonies versus the radiation dose used (Hall, 1988). We used this traditional assay for *in vitro* radioresistance to evaluate isogenic osteogenic sarcoma cell lines that differ only in their pRb expression: the SAOS-2 cell line, which does not express a functional pRb protein (Huang *et al.*, 1988), clonal derivates transfected with the expression vector used throughout our experiments and transfected clonal isolates that express pRb, SAOS-2 (Rb1)/#84 and SAOS-2 (Rb2)/#12 [clones #84 and #12, respectively, in Fung *et al.* (1993)]. The characterization of these transfected derivatives expressing pRb, SAOS-2 (Rb1)/#84 and SAOS-2 (Rb2)/#12, has been published previously (Fung *et al.*, 1993). They express an pRb protein that is phosphorylated appropriately through the cell cycle and binds E2F and E1A. Clonogenic survival curves of SAOS-2, SAOS-2 (Rb1)/#84 and SAOS-2 (Rb2)/#12 demonstrated increased radioresistance in the cells expressing pRb (Figure 1A). Unappreciated by the colony counts reported in this figure are the striking morphological differences between pRb-expressing cells that survived irradiation and irradiated cells lacking pRb. SAOS-2 cells were of roughly uniform size and were clustered in proliferating colonies following irradiation. In contrast, irradiation of SAOS-2 (Rb1)/#84 and SAOS-2 (Rb2)/#12 resulted in the appearance of a new cellular morphology entirely absent from the unirradiated samples as well as from the irradiated SAOS-2 population. In addition to colonies sharing a morphology similar to that observed in proliferating SAOS-2 cells, large, flat cells reminiscent of the growth-arrested SAOS-2 cells seen after pRb transfection (Hinds *et al.*, 1992) were seen throughout the culture as non-proliferating, single cells (Figure 1B).

pRb promotes cellular viability after ionizing radiation exposure

Clonogenic survival assays revealed an increased number of non-clonogenic cells surviving radiation treatment in the pRb-expressing cell lines compared with the cell lines lacking pRb. We therefore sought to quantitate cell viability at several doses and times following radiation exposure. We used a trypan blue exclusion assay to quantify the radiation-induced cell death of SAOS-2 and transfected clonal SAOS-2 isolates. Viability was measured 1, 2, 3 and 6 days after various doses of ionizing radiation. Both SAOS-2 expressing pRb and SAOS-2 lacking pRb showed a time- and dose-dependent decrease in viability following ionizing radiation; however, at all times and doses tested the viability of SAOS-2 (Rb1)/#84 and SAOS-2 (Rb2)/#12, which express pRb, was greater than that of SAOS-2 and SAOS-VEC.4, which express no pRb (Figure 2 and data not shown).

pRb protects osteosarcoma cells from radiation-induced apoptosis

We used several different techniques to test the hypothesis that a difference in susceptibility to apoptosis was

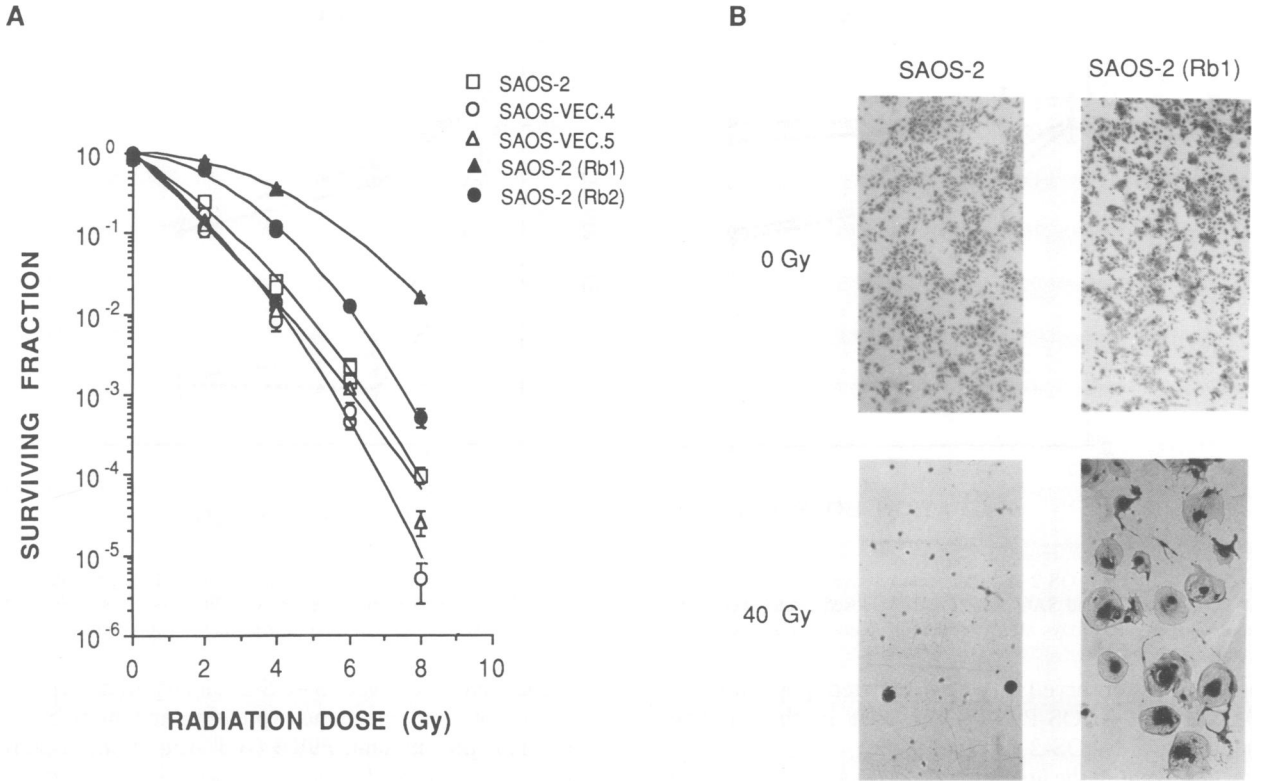

Fig. 1. (**A**) Clonogenic radiation survival curves of SAOS-2 and transfected derivatives. Clonogenic survival assays were performed on SAOS-2, SAOS-VEC.4 and SAOS-VEC.5, all of which lack pRb expression, as well as on SAOS-2 (Rb1)/#84 and SAOS-2 (Rb2)/#12, which express pRb. The number of colonies arising after the specified doses of X-rays was assessed 3 weeks after radiation treatment. Where error bars are not evident, they are smaller than the size of the symbol representing the data point. (**B**) Cellular morphology of SAOS-2 and SAOS-2 (Rb1)/#84 before and after radiation exposure. Equal numbers of cells were plated and photographed prior to radiation treatment or 3 weeks following a radiation dose of 40 Gy. Cells were fixed, stained with methylene blue and photographed at 20× magnification.

Table I. Subdiploid populations in irradiated SAOS-2 derivatives

Radiation dose	Percent subdiploid population			
	SAOS-2	SAOS-VEC.5	SAOS (Rb2)/#12	SAOS (Rb1)/#84
0 Gy	3.2 ± 2.4	5.6 ± 0.8	0.5 ± 0.1	0.16 ± 0.1
8 Gy	13.1 ± 0.7	19.6 ± 0.8	4.1 ± 1.6	1.5 ± 0.4
20 Gy	20.8 ± 1.8	23.8 ± 0.8	5.5 ± 0.7	2.2 ± 0.5

Cell lines were irradiated with the indicated dose of ionizing radiation, harvested 3 days later and subjected to flow cytometry analysis. DNA content was displayed on a log-scale histogram and the percent of cells with less than a 2*n* complement of DNA was calculated. Each value is the mean ± SD of two independent experiments.

associated with the observed pRb-dependent difference in viability following irradiation. Apoptotic cells undergo a series of sequential events in which nuclear chromatin fragmentation is followed by a gradual loss of membrane integrity (Nicoletti *et al.*, 1991; Darzynkiewicz *et al.*, 1992; Dive *et al.*, 1992). Chromatin fragmentation results in cells with a subdiploid complement of DNA (Nicolleti *et al.*, 1991), a process which we have quantified by FACS analysis. Evaluation of SAOS-2, SAOS-2 (Rb1)/#84 and SAOS-2 (Rb2)/#12 cells 1, 2, 3 and 6 days following various doses of ionizing radiation demonstrated an increased subdiploid population emerging 2 days after irradiation, and increasing in a time- and dose-dependent manner (Table I and data not shown). When quantitated at 2, 3 and 6 days, a larger subdiploid population was evident in cells lacking pRb compared with that observed in cells expressing pRb (Table I and data not shown). Interestingly, we observed that the presence of cells with a subdiploid DNA content was diminished in isolates expressing pRb even in the absence of irradiation, suggesting that pRb expression in these cells may protect them from spontaneously occurring apoptosis as well (Table I). To confirm that irradiation of these cell lines resulted in apoptosis, terminal deoxynucleotidyl transferase (TdT) was utilized to document the DNA fragmentation characteristic of this mode of cell death. SAOS-2, SAOS-2 (Rb1)/#84 and SAOS-P9RB.1 cells were treated with 0 or 20 Gy of ionizing radiation and evaluated 3 days after exposure, a time at which radiation-induced DNA double-strand breaks have been repaired (Elkind *et al.*, 1965; Belli and Shelton, 1969; Olive *et al.*, 1991; Story *et al.*, 1994). As indicated in Figure 4, evidence of

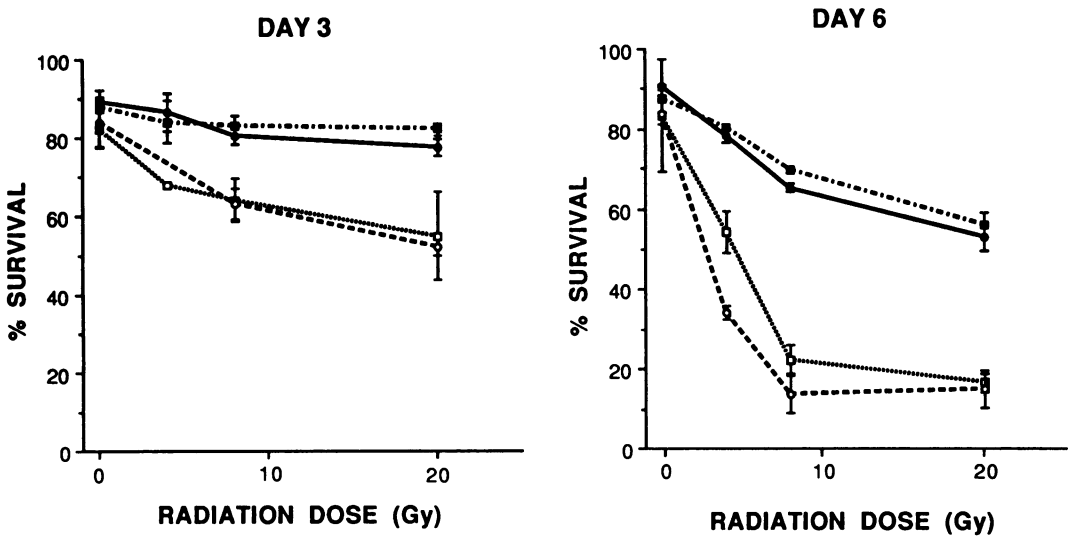

Fig. 2. Viability of SAOS-2 and transfected derivatives after radiation treatment. SAOS-2 (open square), SAOS-VEC.4 (open circle), SAOS-2 (Rb1)/#84 (closed square) and SAOS-2 (Rb2)/#12 (closed circle) were exposed to 0, 4, 8 or 20 Gy of ionizing radiation. Viability was assessed by trypan blue exclusion 3 or 6 days after irradiation. Values represent the mean and error bars represent the SD of three independent experiments.

apoptosis is observed in the irradiated populations of SAOS-2 and SAOS-P9RB.1, but not in the irradiated population of SAOS-2 (Rb1)/#84.

A late event in the apoptotic process is increased plasma membrane permeability, which alters the cellular uptake of DNA-binding fluorochromes, such as Hoechst 33342 (Dive *et al.*, 1992). We treated cells with Hoechst 33342 and used a multiparameter flow cytometry assay to quantitate the apoptotic response induced by radiation (Dive *et al.*, 1992). After the exclusion of dead cells based on their intensely positive staining with propidium iodide, two populations are clearly delineated on a contour plot of Hoechst fluorescence versus forward angle light scatter. Viable cells show low Hoechst fluorescence and high forward light scatter (Figure 3A, 'V'), while apoptotic cells show high Hoechst fluorescence and low forward light scatter (Figure 3A, 'A') indicative of their shrinking cell size and cytoplasmic volume (Gregory *et al.*, 1991, and Figure 3A). We used this assay to quantify apoptotic cells, as shown in Figure 3. Figure 3A shows two-dimensional contour plots of SAOS-VEC.5 and SAOS-2 (Rb1)/#84 cells 3 days after exposure to 0, 8 or 20 Gy of ionizing radiation. A radiation dose of 8 or 20 Gy induced 21 and 29% apoptotic cells, respectively, in SAOS-VEC.5, but only 3 and 6% apoptotic cells, respectively, in SAOS-2 (Rb1)/#84. We sought to confirm that population 'A' (Figure 3A) represented apoptotic cells by examination of nuclear chromatin morphology. Cells from the viable (V) and apoptotic (A) populations were sorted by FACS and stained with diamidinophenylindole (DAPI) (Darzynkiewicz *et al.*, 1992). When viewed in a fluorescence microscope, apoptotic cells were unequivocally identified by their condensed or fragmented chromatin, as shown in Figure 3B.

We used this flow cytometry assay to evaluate the radiation-induced apoptotic response of a variety of cell lines. A representative experiment shown in Figure 3C demonstrates that SAOS-2 (RB1)/#84 and SAOS-2 (Rb2)/#12 underwent significantly less radiation-induced apoptosis than the untransfected SAOS-2 cell line, five independently derived SAOS-2 clones transfected with vector alone and one clone expressing mutant pRb (Figure 3C). This pRb mutant, P9RB (A.T'Ang *et al.*, in preparation) does not bind several known binding partners of pRb, including E2F, SV40 large T antigen and E1A, and fails to suppress cellular growth (A.T'Ang *et al.*, in preparation). The transfected derivative SAOS-P9RB.2, which expresses the mutant pRb (data not shown), exhibited 6 and 19% apoptotic cells after exposure to 8 or 20 Gy, respectively, a degree of apoptosis greater than that exhibited by either of the pRb-expressing clones. The reduction in apoptosis evident in pRb-expressing transfectants was seen after treatment with either 8 or 20 Gy of ionizing radiation. Similar results were obtained in four separate experiments with SAOS-2, SAOS-2 (Rb1)/#12 and SAOS-2 (Rb2)/#84 performed 1, 2 and 3 days following radiation exposure (Figure 3 and data not shown).

Ionizing radiation induced a G_2 arrest in all transfected SAOS-2 derivatives.

The role of pRb in controlling cell cycle progression from G_1 to S (Buchkovich *et al.*, 1989; Goodrich *et al.*, 1991) led us to ask whether pRb's function in protecting cells from apoptosis would be accompanied by an altered distribution of irradiated cells within the cell cycle. One day after X-ray exposure, SAOS-2 and SAOS-VEC.5 cells arrested in G_2, manifested by an increase in the proportion of cells in G_2 phase and a modest decrease in the proportion of cells in S phase (Table II). However, these cells failed to show a dramatic reduction in S phase accompanied by an increase in G_1 phase characteristic of a G_1 arrest. The absence of a G_1 arrest is consistent with the lack of wt p53 expression in SAOS-2 and SAOS-VEC.5 cells (Huang *et al.*, 1988, and data not shown). The G_2 arrest we observed has been documented in numerous cell types and is a dose-dependent response to radiation, increasing in duration with higher doses of radiation (Terasima and Tolmach, 1963; Leeper *et al.*, 1972). Three days after radiation treatment, cells irradiated at 20 Gy were still

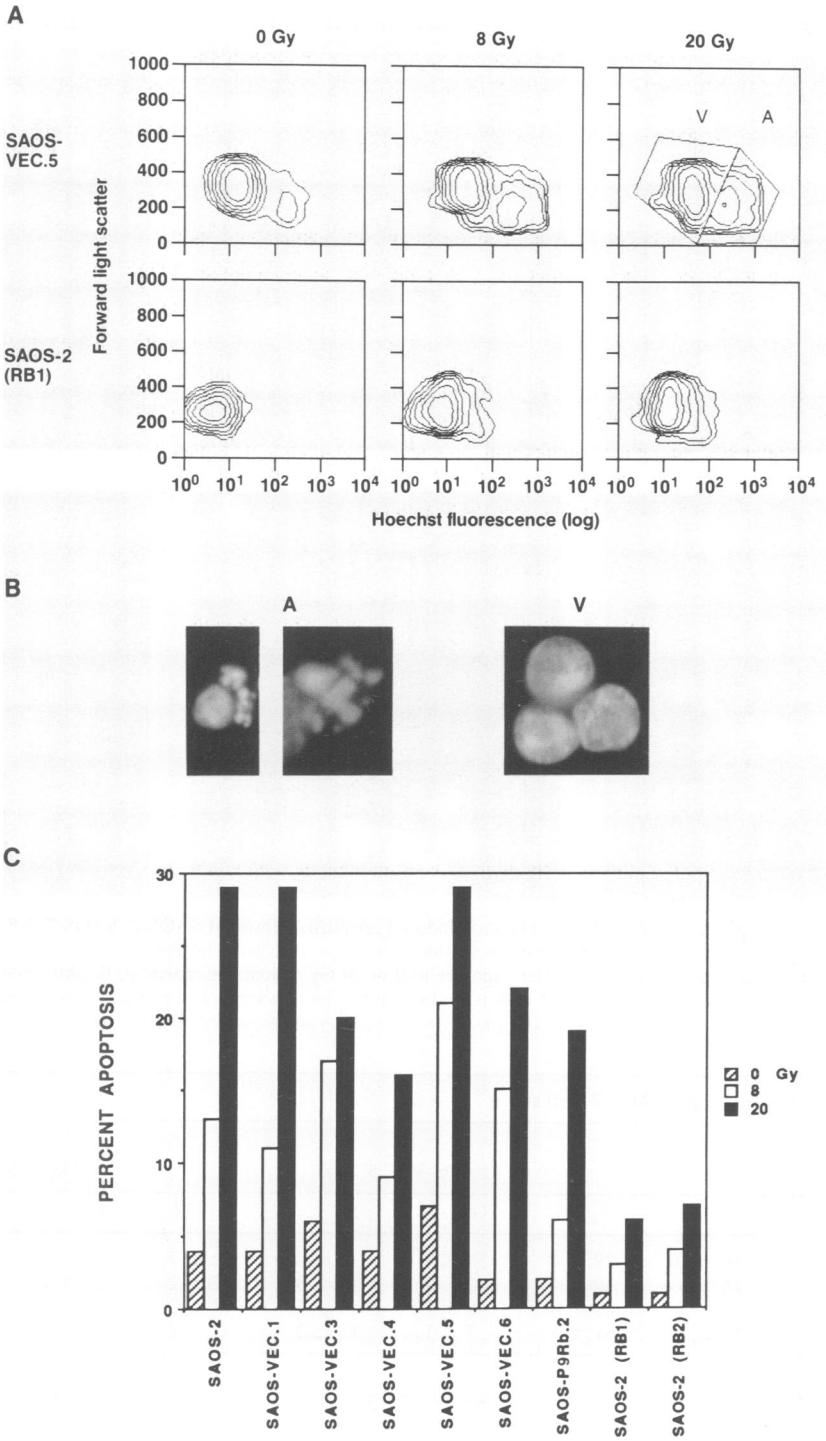

Fig. 3. Radiation-induced apoptosis of SAOS-2 and transfected derivatives. (**A**) Apoptosis was quantified utilizing a FACS two-dimensional contour plot of forward light scatter versus fluorescence of the DNA-binding fluorophore Hoechst 33342 (Dive *et al.*, 1992; Fairbairn *et al.*, 1993). Viable cells display high forward light scatter and low Hoechst 33342 fluorescence (population 'V'), while apoptotic cells display low forward light scatter and high Hoechst 33342 fluorescence (population 'A'). (**B**) Confirmation that populations V and A represented viable and apoptotic populations, respectively, was sought by cell sorting followed by staining with the DNA-binding fluorochrome DAPI. Sorted cells containing fragmented chromatin indicative of apoptosis are shown. (**C**) A representative experiment quantifying radiation-induced apoptosis in SAOS-2 and transfected derivatives. SAOS-2, five independent clones expressing vector alone (SAOS-VEC.1, SAOS-VEC.3, SAOS-VEC.4, SAOS-VEC.5, SAOS-VEC.6), one clone expressing mutant pRb (SAOS-P9Rb.2) and two clones expressing wt pRb [SAOS-2 (Rb1)/#84 and SAOS-2 (Rb2)/#12], were exposed to 0, 8 or 20 Gy of X-rays and subjected to FACS analysis 3 days after treatment. Hatched bars represent unirradiated samples; open bars represent cells irradiated at 8 Gy; solid bars represent cells irradiated at 20 Gy.

arrested in G_2 (Table II). However, 3 days after lower doses of radiation, 4 or 8 Gy, the G_2 arrest began to resolve, leading to a decrease in the G_2/M population and a rebound of the G_1 population (Table II). Likewise, the cell lines SAOS-2 (Rb1)/#84 and SAOS-2 (Rb2)/#12 exhibited a radiation-induced G_2 arrest in the absence of

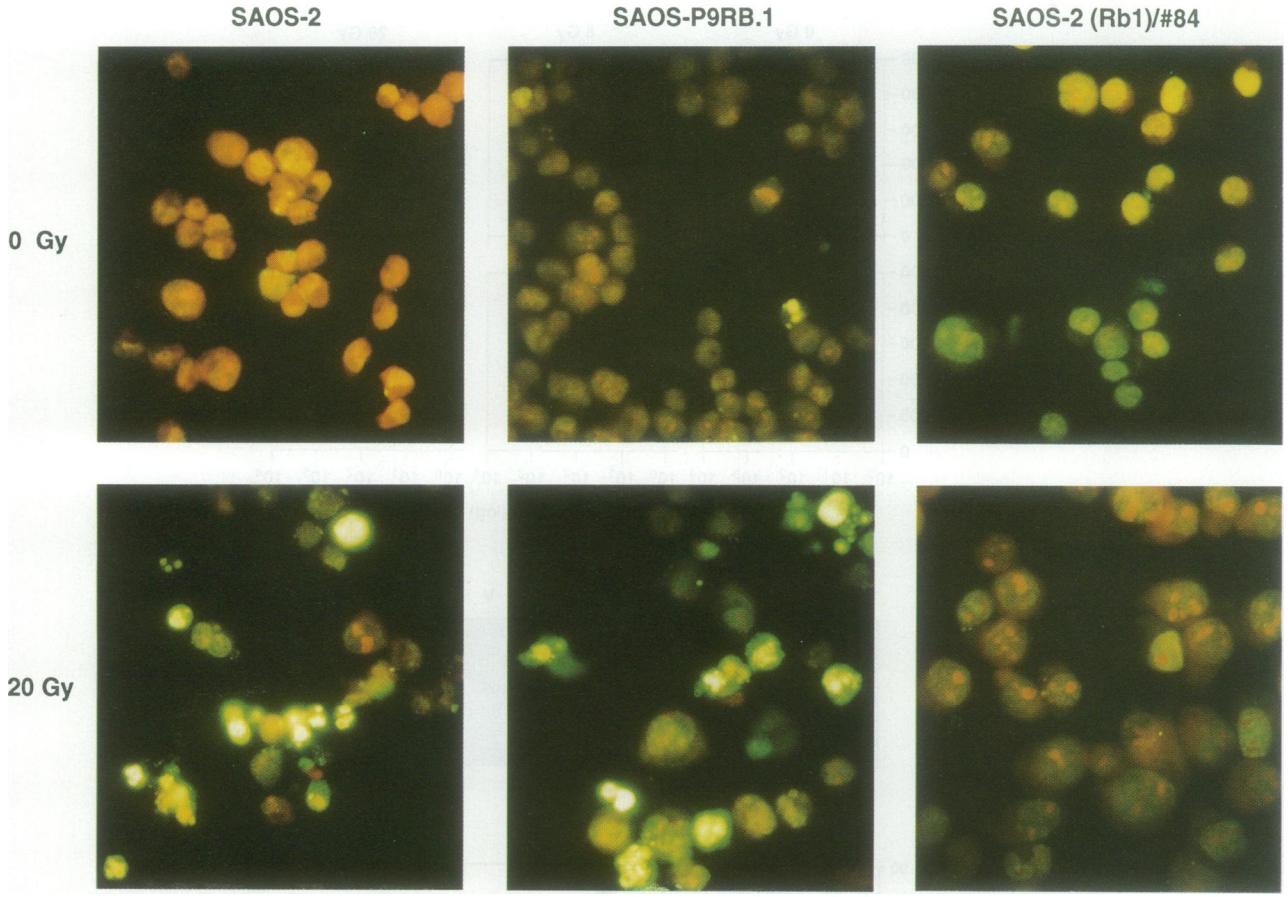

Fig. 4. SAOS-2 cells expressing wt pRb are protected from radiation-induced apoptosis. Unsorted SAOS-2, SAOS-P9RB.1 and SAOS-2 (Rb1)/#84 cells were labeled with digoxigenin-dUTP using terminal deoxynucleotide transferase, detected with fluorescein-conjugated anti-digoxigenin antibody fragments and visualized by fluorescent microscopy 72 h after exposure to 0 or 20 Gy of ionizing radiation. Brightly fluorescent nuclei with apoptotic bodies indicate apoptosis. Cells are counterstained with propidium iodide and photographed at 100× magnification.

Table II. Cell cycle distribution of irradiated SAOS-2 derivatives

Day 1									
	SAOS-2			SAOS-VEC.5			SAOS-2 (Rb1)/#84		
Radiation dose (Gy)	G_1	S	G_2+M	G_1	S	G_2+M	G_1	S	G_2+M
0	55 ± 6	16 ± 10	29 ± 4	54 ± 8	14 ± 2	32 ± 7	48 ± 5	14 ± 4	37 ± 3
4	25 ± 4	16 ± 6	59 ± 4	12 ± 2	15 ± 3	72 ± 2	23 ± 16	12 ± 1	64 ± 16
8	8 ± 4	9 ± 5	83 ± 8	8 ± 2	14 ± 1	71 ± 2	8 ± 3	12 ± 7	80 ± 11
20	7 ± 2	12 ± 3	81 ± 5	7 ± 1	17 ± 2	72 ± 8	9 ± 3	20 ± 3	71 ± 5
Day 3									
	SAOS-2			SAOS-VEC.5			SAOS-2 (Rb1)/#84		
Radiation dose (Gy)	G_1	S	G_2+M	G_1	S	G_2+M	G_1	S	G_2+M
0	61 ± 7	11 ± 2	28 ± 5	60 ± 2	13 ± 1	27 ± 1	65 ± 8	8 ± 1	26 ± 8
4	38 ± 2	13 ± 2	49 ± 1	41 ± 1	13 ± 1	45 ± 1	50 ± 2	11 ± 2	39 ± 2
8	29 ± 3	13 ± 3	58 ± 2	21 ± 2	12 ± 2	67 ± 2	42 ± 1	11 ± 2	47 ± 1
20	16 ± 5	7 ± 1	77 ± 5	6 ± 4	5 ± 2	89 ± 4	8 ± 3	5 ± 2	86 ± 3

SAOS-2, SAOS-VEC.5 and SAOS-2 (Rb1)/#84 were treated with the indicated doses of ionizing radiation. One or 3 days after radiation exposure, the cells were harvested and subjected to flow cytometry analysis to determine the cell-cycle profiles. Each value is the mean ± SD of two to five independent experiments.

a G_1 arrest (Table II and data not shown). Within the first 24 h after irradiation, these cell lines failed to show an increased G_1 phase and a marked reduction in S phase, as would result from a G_1 arrest. The kinetics of these cell cycle modulations after radiation exposure did not differ substantially between SAOS-2 (Rb1)/#84, SAOS-2 (Rb2)/#12, SAOS-2 and SAOS-VEC.5, except for one notable difference. Three days after exposure to 4 or 8 Gy, the

rebound seen in the G_1 population of SAOS-2 (Rb1)/#84 and SAOS-2 (Rb2)/#12 was consistently greater than that seen in SAOS-2 and SAOS-VEC.5.

Aphidicolin prevented cell cycle progression into S phase but did not protect osteosarcoma cells from apoptosis

Although the SAOS-2 transfectants exhibited no radiation-induced G_1 arrest, we investigated whether the ability of pRb to modulate apoptosis might nevertheless be associated with the role of pRb in mediating the G_1/S transition during cell cycle progression. We used aphidicolin to prevent SAOS-2 cells from progressing into S phase following irradiation, and asked whether holding the cells at the G_1/S transition could diminish the apoptotic response. Aphidicolin inhibits the activity of DNA polymerase α, and thereby prevents passage through S phase (Haraguchi *et al.*, 1983; Aoshima *et al.*, 1984). As seen in Figure 5A, no difference in the percentage of apoptotic cells was seen between the untreated and the aphidicolin-treated populations of SAOS-2 or SAOS-2 (Rb1)/#84. To confirm that aphidicolin blocked passage through S phase over the 48 h incubation following irradiation, cell cycle analysis was performed on the same cells assayed for apoptosis. Figure 5B shows that aphidicolin successfully prevented the majority of cells from exiting G_1. In the absence of aphidicolin treatment, 56% of unirradiated SAOS-2 cells were in G_1 compared with 8% of irradiated SAOS-2 cells. In the presence of aphidicolin, both unirradiated and irradiated SAOS-2 populations showed a comparable accumulation in the G_1 phase of the cell cycle (76 and 71%, respectively). Thus, artificially keeping SAOS-2 cells at the G_1/S transition and preventing their progression into S phase failed to mimic the protection from apoptosis produced by pRb.

pRb expression confers complete protection from radiation-induced apoptosis in a transient transfection assay

Transfection of *RB* into SAOS-2 cells typically results in a growth arrest in G_0/G_1 (Shew *et al.*, 1990; Goodrich *et al.*, 1991; Qin *et al.*, 1992). The transfected isolates SAOS-2 (Rb1)/#84 and SAOS-2 (Rb2)/#12 are unusual in their capacity to continue proliferating while expressing pRb. We therefore pursued an independent approach to extend our findings, using a transient transfection assay to examine pRb function in SAOS-2. In these transfections, we used recombinant molecules (pβActRB and pβActP9RB) in which *RB* or a mutant *RB* (P9RB; A.T'Ang *et al.*, in preparation) were cloned into the vector pβAct-vector (Fung *et al.*, 1993) and transcription of these genes was regulated by the human β-actin promoter. The pCMVCD20 plasmid encoding the surface antigen CD20 was co-transfected into SAOS-2 cells with the expression vectors specified in Figure 6. We have previously demonstrated that transfection of SAOS-2 using the identical recombinant pβActRB, pβActP9RB and pCMVCD20 expression plasmids resulted in pRb and comparable mutant pRb expression 48–72 h following transfection (Iavarone *et al.*, 1994; A.T'Ang *et al.*, in preparation). The CD20 cell-surface marker can be used to demarcate cells expressing transfected proteins, using flow cytometry to detect staining with a fluorescein isothiocyanate (FITC)-conjugated anti-CD20 antibody (Zhu *et al.*, 1993). Within a given sample, CD20-positive and CD20-negative cells were analyzed separately as representatives of transfected and untransfected cells, respectively. The percentage of apoptotic cells in the CD20-positive and CD20-negative populations was quantified by FACS as described in Figure 3A.

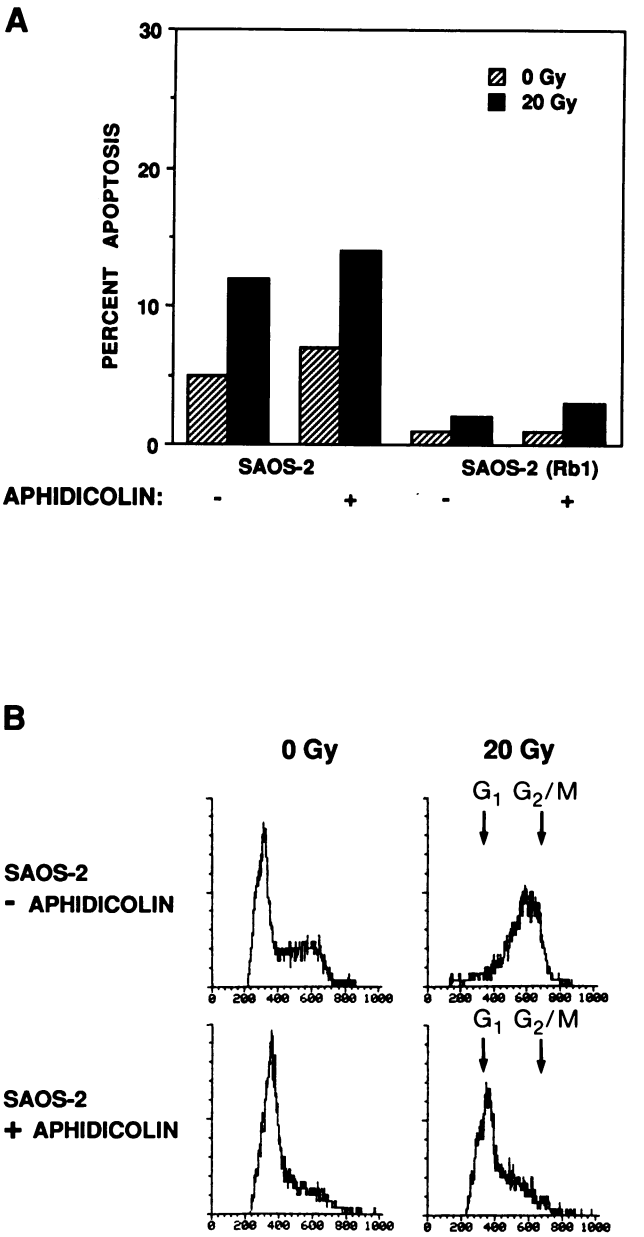

Fig. 5. Aphidicolin treatment does not alter the degree of radiation-induced apoptosis. (**A**) SAOS-2 cells and SAOS-2 (Rb1)/#84 cells were exposed to 0 or 20 Gy of X-rays. Aphidicolin treatment was continuous for 48 h, initiated immediately after irradiation. Flow cytometry analysis to determine the percentage of apoptotic cells was performed 48 h after radiation treatment. Hatched bars represent unirradiated samples; solid bars represent irradiated samples. (**B**) The same samples analyzed for apoptosis in (A) were incubated for an additional hour in Hoechst 33342 to determine their cell cycle profiles. DNA histograms are shown for aphidicolin-treated and untreated SAOS-2 samples after treatment with 0 or 20 Gy of ionizing radiation.

We used this assay to evaluate SAOS-2 cells transfected with pRb, mutant pRb, the parent expression vector

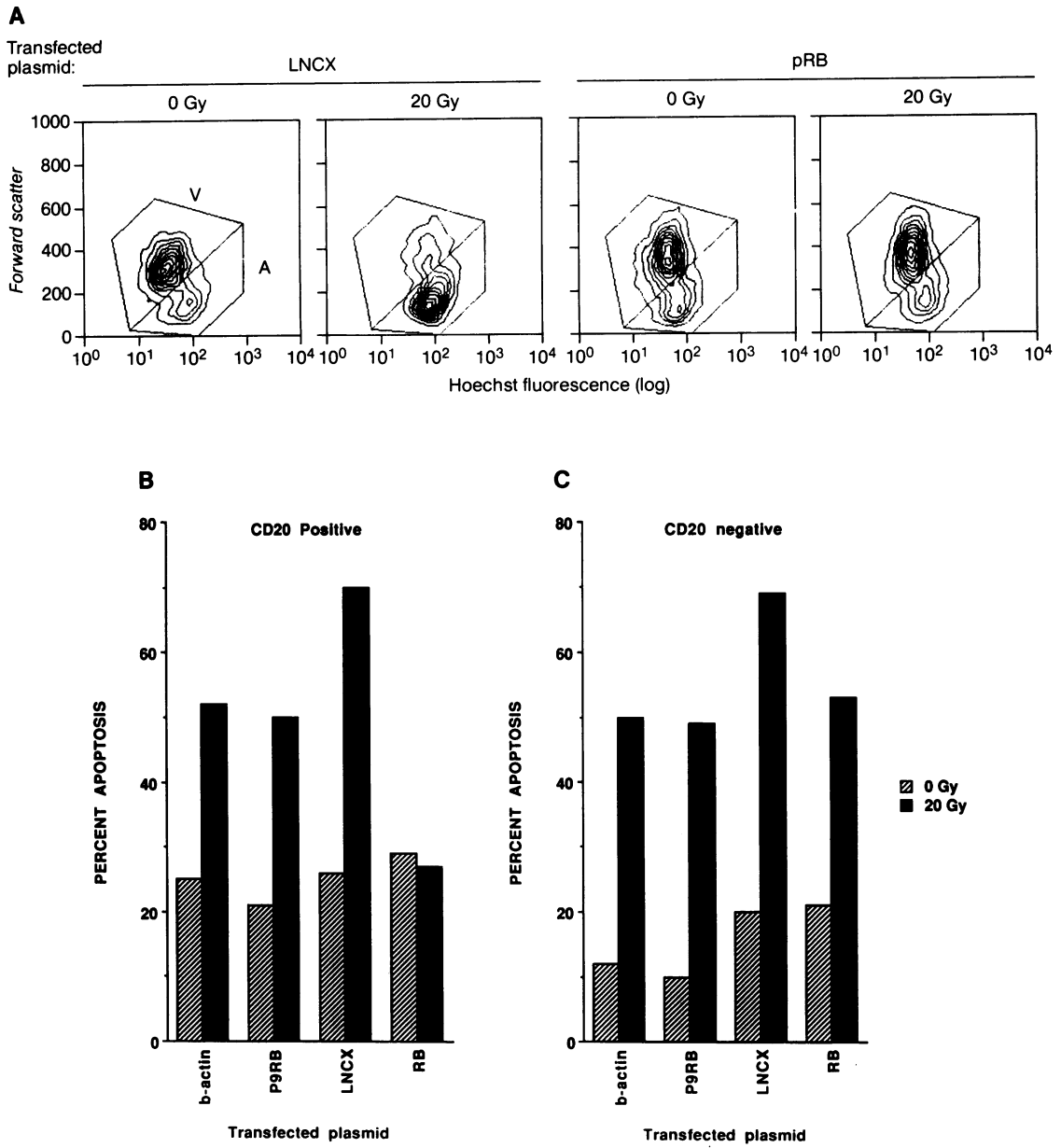

Fig. 6. pRb-mediated protection from apoptosis in a transient transfection assay. (**A**) SAOS-2 cells were transfected with 10 μg of the indicated plasmid plus 5 μg of pCMVCD20. Two days after transfection, cells were exposed to either 0 or 20 Gy of ionizing radiation. Three days after radiation treament, cells were harvested for flow cytometry analysis (see Materials and methods for details). Transfected cells, recognized by their expression of the co-transfected CD20 cell-surface marker, were subjected to a multiparameter assay delineating viable cells (population V) and apoptotic cells (population A). (**B**) The percentage of apoptotic cells in irradiated and irradiated samples was determined 2 days (for β-actin, RB and P9RB) or 3 days (for LNCX and *RB*) after radiation treament. CD20-positive cells were subjected to the multiparameter assay shown in (A). (**C**) CD20-negative cells from the same unirradiated and irradiated samples shown in (B) were analyzed for apoptosis. The data represent three independent experiments in which RB transfected cells were compared with one of the three illustrated controls, β-actin, P9RB or LNCX. Hatched bars represent unirradiated samples; solid bars represent irradiated samples.

pβActvector or the expression vector pLNCX. A marked radiation-induced apoptotic response was evident in CD20-positive and CD20-negative SAOS-2 cells transfected with the control vector pLNCX. Three days after treatment with 20 Gy, these control samples contained 69 and 70% apoptotic cells, respectively (Figure 6B and C). A similar increase in apoptosis following radiation exposure was seen in CD20-negative SAOS-2 cells transfected with pβActRB (Figure 6C). In contrast, we found no increase in apoptosis of CD20-positive cells transfected with pβActRB after treatment with 20 Gy of ionizing radiation (Figure 6B). Three days following irradiation, the percentage of apoptotic cells remained virtually unchanged at 27 ± 3%, a baseline due in large part to transfection-associated toxicity. When co-transfected with pβActRB, the CD20-positive SAOS-2 population showed no change in apoptosis in any of three independent experiments, evaluated either 2 or 3 days after irradiation. Irradiation of samples transfected with either pβActP9RB, the parent pβActvector or the pLNCX vector consistently demonstrated an increase in apoptotic cells in the CD20-positive and CD20-negative populations (Figure 6B and C).

Discussion

In this study, we demonstrate a novel pRb function: pRb-mediated suppression of apoptosis. Our investigation utilizes a pRb-deficient cell line to study both transiently and stably transfected derivatives expressing pRb. We initially noted that the expression of pRb was associated with the persistence of non-proliferating cells 3 weeks after treatment with several different doses of ionizing radiation. By quantitating cellular viability, we showed that ionizing radiation induces less cell death in cells that expressed pRb. Furthermore, we demonstrated that this pRb-dependent reduction in cell death is due to a pRb-mediated inhibition of apoptosis. This new function of the retinoblastoma protein has important implications for normal development and neoplastic transformation, both of which require a tightly controlled balance between proliferation and apoptosis.

Several recent lines of investigation suggest that pRb may inhibit apoptosis. *RB*-deficient mice exhibit massive apoptosis not observed in normal mice (Clarke *et al.*, 1992). These mice die in mid- to late gestation with extensive apoptosis evident in the nervous system. Inappropriate apoptosis has also been well documented in the developing ocular lens of these Rb-deficient mice (Morgenbesser *et al.*, 1994). These observations suggesting a role for pRb in mediating apoptosis have been extended in a recent study of E2F-induced apoptosis (Wu and Levine, 1994). When constitutively expressed in a mouse embryo fibroblast cell line, the transcription factor E2F-1 was capable of inducing apoptosis. E2F is inactive when present in a complex with pRb (Nevins, 1992), which is consistent with the hypothesis that pRb may protect cells from apoptosis, possibly by sequestering E2F. Studies of several viral oncoproteins provide additional evidence that pRb suppresses apoptosis. Human papilloma viruses encode a transforming protein, E7, which binds and inactivates pRb (Dyson *et al.*, 1989, 1992). E7 has recently been shown to mediate rapid cell death reminiscent of apoptosis (White *et al.*, 1994). A second viral oncoprotein which binds and inactivates pRb, adenovirus E1A, also induces cell death, which Rao *et al.* (1992) have demonstrated occurs through apoptosis. Mutational analysis of E1A revealed that it forms a complex with pRb via the E1A sequences that are required for both transformation and induction of apoptosis. These findings suggest a link between the ability of these transforming proteins to bind pRb and their function in inducing apoptosis. This link is strongly supported by our evaluation of the pRb-mutant, P9Rb, which fails to bind E1A and E2F (A.T'Ang *et al.*, in preparation) and lacks the capacity to inhibit radiation-induced apoptosis. Our study introduces pRb as a regulator of apoptosis, and raises the possibility that E1A, E7 and E2F increase susceptibility to apoptosis by specifically interfering with the protective function of pRb.

Our observation that pRb suppresses apoptosis was evident whether exogenous pRb was expressed stably or transiently. The stably transfected cell lines allowed us to examine the ultimate effect of pRb expression on cell survival, as measured by sustained proliferative capacity. Transiently transfected cells complemented our line of investigation by allowing us to analyze non-clonal populations of pRb-expressing SAOS-2 cells.

The ability of pRb to protect cells from apoptosis does not require wt p53 expression. This result is consistent with recently published studies of the developing mouse lens in which loss of p53 attenuates, but does not eliminate, the inappropriate apoptosis associated with a lack of pRb expression (Morgenbesser *et al.*, 1994; Pan and Griep, 1994). There are clearly p53-dependent and independent mechanisms of apoptosis in response to DNA damage (Lowe *et al.*, 1993a; Strasser *et al.*, 1994). Transfection of the viral oncogene E1A into fibroblasts triggers p53-dependent apoptosis in response to low doses of DNA-damaging agents, but can induce p53-independent apoptosis in response to higher doses of the same agents (Lowe *et al.*, 1993a). In SAOS-2 cells which lack functional p53, we observed apoptosis following relatively high doses of ionizing radiation and long incubation times. The apoptotic response after high radiation doses and long incubation periods following treatment has also been studied in a variety of other cell types, including p53-deficient cells (Yonish-Rouach *et al.*, 1991; Radford *et al.*, 1994; Strasser *et al.*, 1994). In some cell types, apoptosis documented by morphology and DNA laddering was observed several days after radiation doses >2-fold higher than those used in our study (Radford *et al.*, 1994).

The mechanism by which pRb protects cells from radiation-induced apoptosis is not yet clear. It has been hypothesized that pRb may prevent apoptosis by promoting a quiescent state in which the cells are less susceptible to apoptosis (Clarke *et al.*, 1992). Consistent with this hypothesis is our finding that associated with the suppression of apoptosis that occurs following the transient expression of pRb in SAOS-2 cells there is an accumulation of cells in G_0/G_1 (Shew *et al.*, 1990; Goodrich *et al.*, 1991; Qin *et al.*, 1992; Iavarone *et al.*, 1994). In addition, the only significant difference in cell cycle distribution following irradiation between our SAOS-2 derivatives lacking or stably expressing pRb was the greater rebound in the number of cells in G_0/G_1 phase after resolution of the G_2 arrest. One interpretation of this difference in cell cycle distribution is that pRb facilitates accumulation of cells in G_0/G_1 phase after radiation exposure and therefore decreases cellular susceptibility to apoptosis. Further studies using synchronized cells may elucidate the exact interplay between pRb's roles in the cell cycle and in apoptosis.

Aphidicolin may have been expected to protect SAOS-2 cells from radiation-induced apoptosis, since it arrests cell cycle progression at the G_1/S border (Haraguchi *et al.*, 1983; Aoshima *et al.*, 1984). The inability of aphidicolin to suppress apoptosis, evident from our results, points to a more complex mechanism for the protective effect of pRb. Whereas aphidicolin specifically inhibits DNA polymerase α, pRb interacts with many cell cycle regulators, including E2F, cyclin D, Id-2 and c-myc (Chellappan *et al.*, 1991; Rustgi *et al.*, 1991; Helin *et al.*, 1992; Kaelin *et al.*, 1992; Dowdy *et al.*, 1993; Iavarone *et al.*, 1994). Simply arresting cell cycle progression by inhibiting DNA polymerase α does not recreate the complex molecular cascades produced by pRb expression.

The mechanism by which pRb protects cells from apoptosis may influence the proliferative capacity of these surviving cells following radiation exposure. Apoptosis measurements, as an endpoint of radiation cytotoxicity,

depart from the traditional assay for *in vitro* radiation resistance, the clonogenic assay. The clonogenic survival curve is the most widely used assay for *in vitro* radiation resistance and relies on measuring the fraction of cells that are able to proliferate after exposure to specific doses of radiation (Hall, 1988). Our results show increased clonogenic survival in the SAOS-2 transfected derivatives expressing pRb. The relationship between the observed protection from apoptosis and increased clonogenic radioresistance remains unclear; however, several mechanisms are possible. Cells protected from apoptosis may themselves proliferate and contribute directly to increased clonogenic radioresistance. Alternatively, these cells may remain in G_0/G_1, but support the proliferation of other cells, perhaps by secreting growth factors or extracellular matrix components.

During development, one function of apoptosis is the elimination of cells that could proliferate inappropriately. Apoptosis may protect the organism from cells that would otherwise divide in the setting of growth-arrest signals such as absent growth factors, DNA damage or nutrient withdrawal. It is not surprising that apoptosis and proliferation are regulated by some of the same genes, as evident from the study of *myc* (Askew *et al.*, 1991; Evan *et al.*, 1992). Deregulated c-myc signals cellular proliferation. However, in cells from which the mitogenic stimulus has been removed, deregulated c-myc induces apoptosis (Evan *et al.*, 1992). The role of pRb in controlling cell cycle progression and apoptosis parallels the dual functions of c-myc. The presence of pRb and its antiproliferative function may facilitate the growth arrest typically associated with DNA damage (Slebos *et al.*, 1994; White *et al.*, 1994). In the absence of pRb's antiproliferative effect, damaged cells may apoptose after receiving the signal to proceed inappropriately through the cell cycle.

The regulatory coupling of apoptosis and proliferation, as evidenced by c-myc function and our current study of pRb, has important implications for tumor biology. As long as this coupling is intact, the genetic alteration allowing inappropriate proliferation of the tumor cell will also increase its susceptibility to demise by apoptosis. Loss of this coupling by alteration of genes important for either apoptosis or proliferation may diminish tumor cell death by apoptosis. Diminished tumor cell apoptosis could contribute both to tumor progression by enhancing the survival of cells sustaining DNA damage and to therapeutic resistance by decreasing the efficacy of antineoplastic agents (Lowe *et al.*, 1993a). Thus, the uncoupling of the apoptotic and proliferative regulatory pathways may be a key step in oncogenesis and in determining the responsiveness of tumors to commonly employed modalities of anti-neoplastic therapy.

Materials and methods

Cell culture and radiation treatment

The human osteosarcoma cell line SAOS-2 was obtained from the American Type Culture Collection (ATCC). The pRb-expressing clonal isolates SAOS-2 (Rb1)/#84 and SAOS-2 (Rb2)/#12 have been previously characterized [clones #84 and #12, respectively, in Fung *et al.* (1993)]. All cells were grown in Dulbecco's modified Eagle's media (DMEM) containing 10% fetal bovine serum (FBS; Gibco) supplemented with penicillin and streptomycin. The transfected clones were grown continuously in growth media containing 400 μg of G418 (Geneticin, Gibco) until the day prior to irradiation, at which time the media in all dishes were changed to G418-free culture media. Irradiation was performed at room temperature in a 150 kV Philips X-ray machine at a dose rate of 1.2 Gy/min.

Plasmids and transfection

The plasmids pHuβAPr-1-neo, its *RB*-containing derivative HuβAcpr-1-neo-PQ and a mutant *RB*-containing derivative HuβAcpr-1-neo-PQ (herein referred to as pβActvector, pβActRB and pβActP9RB, respectively) were obtained from Y.-K.T.Fung (Childrens Hospital, Los Angeles, CA; Fung *et al.*, 1993). The plasmid pCMVCD20 was obtained from E.Harlow (Massachusetts General Hospital, Boston, MA; Zhu *et al.*, 1993).

Calcium phosphate transfections were carried out substantially as described previously (Iavarone *et al.*, 1994). A total of 1.8×10^6 cells were plated in 100 mm dishes in 10 ml of complete growth medium the night prior to transfection. Immediately prior to transfection, the medium was changed to 11 ml of complete growth medium supplemented with 25 μM chloroquine. Then 1.4 ml of HEPES buffered saline solution (pH 7.05) was added to an equal volume of DNA/$CaCl_2$ solution and the resulting mixture was immediately added to the above medium (Pear *et al.*, 1993). Medium was changed to fresh complete growth medium without chloroquine 10 h after transfection, and again 24 h after transfection. To generate stable transformants of the SAOS-2 cell line, transfection was followed by a 3 week selection in complete culture medium containing 400 μg/ml of G418.

Viability measurements

A total of 3×10^5 cells were seeded per 10 cm dish in complete tissue culture media. Three days later, the cells were exposed to the indicated dose of ionizing radiation and then incubated at 37°C until harvesting. At specified times following irradiation, adherent and non-adherent cells were pooled, resuspended in phosphate-buffered saline (PBS) and counted in the presence of trypan blue. Viability was assessed by the ability to exclude trypan blue. Values represent the average viability and error bars represent the SD of at least three independent experiments, each counted in duplicate.

Apoptosis assays

SAOS-2 and SAOS-2 (Rb1)/#84 cells were cultured, irradiated and harvested on the designated day as described above. Cells containing a subdiploid DNA content were delineated by FACS (Becton Dickinson FACScan and Lysis II software) as described previously (Nicoletti *et al.*, 1991).

Quantification of apoptotic and viable cells was accomplished by a multiparameter assay measuring forward light scatter and fluorescence of the DNA-binding fluorochromes Hoechst 33342 (Sigma) and propidium iodide (Boehringer Mannheim), as previously described (Dive *et al.*, 1992; Fairbairn *et al.*, 1993). In brief, cells were adjusted to a density of 1×10^6/ml in PBS supplemented with 10% FBS and stained with 5 μg/ml propidium iodide for 30 min at 4°C. After a 2 min pulse with Hoechst 33342 (10 μM) at room temperature, multiparameter analysis of 1×10^4 cells was performed on a FACStar Plus (Becton Dickinson and Lysis II software). Dead cells were excluded on the basis of their intensely positive staining with propidium iodide, and cell debris was excluded by gating forward and side light scatter. On a two-dimensional frequency contour plot of Hoechst fluorescence (log scale) versus forward light scatter (linear scale), two clearly delineated populations could be distinguished: viable cells which displayed low Hoechst fluorescence and high forward light scatter, and apoptotic cells which displayed high Hoechst fluorescence and low forward light scatter. Confirmation of this representation of apoptosis was verified by sorting of the defined populations followed by examination of nuclear chromatin morphology. Cells from the viable (V) and apoptotic (A) populations were sorted, fixed overnight in 70% ethanol at 4°C, stained with 30 μg/ml DAPI (Boehringer Mannheim) for 30 min at 37°C and spun onto glass slides. When viewed in a fluorescence microscope, apoptotic cells were unequivocally identified by their condensed or fragmented chromatin (Darzynkiewicz *et al.*, 1992). Additional apoptosis assays utilized the TdT-labeling technique. T25 flasks of 40% confluent cells were exposed to 0 or 20 Gy of ionizing radiation. Seventy-two hours after irradiation all cells from each flask were collected, washed in PBS and fixed for 30 min in 1.5% paraformaldehyde in PBS. The cells were then washed in PBS, resuspended in 70% ethanol at 4°C for 1 h and cytospins were prepared on poly-L-lysine pre-treated slides. Apoptotic cells were labeled with digoxigenin-conjugated dUTP using TdT (Gavrieli *et al.*, 1992). These cells were detected with fluorescein conjugated anti-digoxigenin

antibody fragments (Oncor, Inc.) and visualized with fluorescent microscopy at 494 nm.

Quantitation of apoptosis in transiently transfected cells utilized co-transfection of an expression vector for the cell-surface protein CD20 (pCMVCD20) with vector alone or *RB* constructs as described above. For FACS analysis, we used a protocol modified from a previously described technique (Zhu *et al.*, 1993). SAOS-2 cells were transfected with 5 μg of pCMVCD20 (when so indicated) and 10 μg of each plasmid indicated in Figure 6. Forty-eight hours after the removal of DNA precipitates, samples were exposed to either 0 or 20 Gy of X-rays. Two or 3 days after radiation treatment, Cell Dissociation Buffer (Gibco BRL) was used to dislodge cells from the dish, and adherent and non-adherent cells were pooled. The cells were pelleted at 1000 *g* and stained with 20 ml of a FITC-conjugated anti-CD20 monoclonal antibody (Beckton-Dickinson), as described previously (Zhu *et al.*, 1993). Following FITC staining, cells were stained with propidium iodide and pulsed with Hoechst 33342 as described above. Data for FACS analysis were collected on 100 000 cells/sample. A gate was set to select CD20-positive cells with FITC staining at least 20-fold brighter than the negative untransfected cells. The number of CD20-positive cells analyzed per sample varied from 900 to 5000. Multiparameter flow cytometry analysis was used to delineate apoptotic and viable cells, as described above.

Cell cycle analysis

Propidium iodide staining was used to delineate cell cycle distribution as described previously (Zhu *et al.*, 1993). The data represent the mean of two to five experiments and their respective SDs.

Aphidicolin treatment was used to block cell cycle progression into S phase. Cells were seeded, incubated and irradiated as described above. Immediately following irradiation, aphidicolin (Boehringer Mannheim, final concentration 3 μg/ml) was added to the appropriate flasks and all flasks were then returned to the incubator. Analysis of apoptosis was performed as described above, with the exception that cells were harvested 2 days post-irradiation. Hoechst-stained samples were incubated for an additional hour at room temperature and cell cycle distribution was determined using Hoechst fluorescence as a measure of DNA content.

Clonogenic radiation sensitivity assays

Cells were plated, grown and irradiated as described above. On the day prior to irradiation, autologous cells were irradiated with 40 Gy and plated in 6-well plates to function as a feeder layer. On the following day, cells for the clonogenic survival curve were seeded as previously described (Sarkar *et al.*, 1993). In brief, after exposure to a specified dose of radiation, cultures were trypsinized, counted and plated at specified concentrations in the wells already containing an autologous feeder layer. These cultures were then incubated for 21 days and colonies of >50 cells were scored. Cell survival measurements were fitted to a linear quadratic mathematical model using the FIT 2.10 program (Fertil and Malaise, 1985; Albright, 1987). Within each of at least two independent experiments, two to four different dilutions were made per radiation dose and each dilution was plated in multiples of six.

Acknowledgements

We thank Martin Haas and Dennis F.Deen for helpful discussion, and Andrew Murray for comments on the manuscript. We thank Jennie Hsu for technical assistance. We thank Norman Albright for his invaluable assistance. This work was supported by the Betz Foundation, National Institutes of Health training grant CA-09215 to the Department of Radiation Oncology, University of California, San Francisco. S.C.K. is supported by grant #PRTA-11 from the American Cancer Society.

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Received on September 13, 1994; revised on November 8, 1994