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Activation of cyclin A-dependent kinases associated with WAF1 degradation during radiation-induced apoptosis

Tei Wei[1,4], Richard T. Williams[1,2,4] and Martin F. Lavin[1,2,3]

[1] Queensland Cancer Fund Research Unit, Queensland Institute of Medical Research, Bancroft Centre, 300 Herston Road, Brisbane, Australia, 4029
[2] Department of Surgery, University of Queensland, Royal Brisbane Hospital, Herston, Brisbane, Australia, 4029
[3] Corresponding author: Tel: 61 7 3362 0341; fax: 61 7 3362 0106; email: martinL@qimr.edu.au
[4] Both authors made an equal contribution to this work.

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Abstract

We describe here the further characterisation of the radiation response of a pair of isogenic Burkitt's lymphoma cell lines which differ significantly in their susceptibility to radiation-induced apoptosis. In both cases a marked inhibition of cyclin A-dependent kinase activity was observed at 4 h post-irradiation which recovered to normal levels in the susceptible line by 12 h but remained inhibited in the resistant cell line. Under these conditions the cellular abundance of $p58^{cyclinA}$ and $p33^{cdk2}$ did not significantly change in the two cell types and there was no evidence for phosphorylation changes in $p33^{cdk2}$ which might account for the activity differences. In parallel with the changes in activity, $p21^{WAF1}$ increased initially in both cell lines, declined in the sensitive cell line as the activity recovered but remained high in the resistant cell line. This appears to be explained by a more rapid turn-over of $p21^{WAF1}$ in the sensitive cell line and an increased association of $p21^{WAF1}$ with cyclin kinase as determined by immunoprecipitation. These results implicate $p21^{WAF1}$ in the regulation of cyclin-dependent kinases during radiation-induced apoptosis, with persistence of induced $p21^{WAF1}$ being associated with a more resistant phenotype.

Keywords: radiation, apoptosis, cyclinA-dependent kinase, $p21^{WAF1}$

Abbreviations: CDKs, cyclin-dependent kinases; CHX, cycloheximide; CPB, citrate phosphate buffer; PBS, phosphate buffered saline

Introduction

The cellular response to external stimuli and subsequent progression into apoptosis is regulated by the complex interplay between components of signal transduction pathways (Kung, 1990; Wagner, 1994; Canman, 1985). Recent experimental evidence points to a central role for a number of radiation responsive and other factors, including p53, $p21^{WAF1}$, bcl-2, bax, bcl-x and PKC isoforms in mediating radiation-induced cell killing (El-Deiry, 1994; Khanna *et al*, 1996).

One of these proteins, p53, confers susceptibility to G_1 arrest or apoptosis in response to irradiation in different cell types (O'Connor *et al*, 1993). On the other hand no G_1 arrest is observed when p53 is mutated, and apoptosis can be prevented under these conditions (Lotem and Sachs, 1993, Bae *et al*, 1995). While p53 is frequently mutated in Burkitt's lymphoma cell lines (Farrell *et al*, 1991), recent evidence suggests that even in the absence of functional protein, cells maintain sensitivity to radiation-induced apoptosis through a p53-independent pathway associated with G_2/M arrest (Allday *et al*, 1995, Han *et al*, 1995, Khanna *et al*, 1996). The radiation signal transduction pathway operating through p53 is now well described (Kastan *et al*, 1991; Dulic *et al*, 1994) being responsible for inducing $p21^{WAF1}$ and GADD45 genes involved in cell cycle control and DNA repair (Fornace *et al*, 1992, Canman *et al*, 1994). $p21^{WAF1}$ binds to and inhibits the activity of cyclin-dependent kinases (Xiong, 1993; Dulic, 1994).

As a consequence substrates such as the retinoblastoma protein are not phosphorylated and inhibition of progression from G_1 to S-phase is observed (Slebos *et al*, 1994). Cyclin kinase activities are also inhibited during S-phase and at the G_2/M transition (Dulic *et al*, 1994, Beamish *et al*, 1996).

Initial characterisation of several Burkitt's lymphoma cell lines revealed that the susceptibility to radiation-induced cell killing varied significantly, and that there was no consistent relationship between sensitivity and p53 status, c-*myc*, bcl-2, bax, or bcl-X expression (Khanna *et al*, 1996, Filippovich *et al*, 1994). This is not all that surprising since it has been shown that proliferating T lymphocytes (Strasser *et al*, 1994, Seki *et al*, 1994) are sensitive to γ-ray induced apoptosis in a p53-independent manner. Furthermore myc-mediated apoptosis shows a requirement for wild-type p53 independent of $p21^{WAF1}$ induction and cell cycle arrest (Wagner, *et al*, 1994). In order to investigate the importance of cell cycle control in susceptibility and resistance to apoptosis we compared the effects of ionizing radiation on cell cycle progression with regulation of cyclin-dependent kinase (CDK) activity. Isogenic Burkitt's lymphoma cell lines, BL30 and BL30(S), were employed as a model system to investigate the activation of CDKs during apoptosis. The expression and subunit association of $p58^{cyclin\ A}$ and $p33^{cdk2}$ and the role of $p21^{WAF1}$, an effector of p53 function, were studied in the regulation of CDK activity during apoptosis induced by radiation.

Results

Cellular response to radiation

The radiation response of the two cell lines used in this study was further characterised. As shown in Figure 1, the BL30 cell line showed hypersensitivity to cell killing in respsonse to 10 Gy of γ-rays with time after irradiation, while the derived cell line, BL30(S), was resistant (65% and 5% apoptosis at 12 h post-irradiation respectively). Propidium Iodide staining and DNA analysis of these Burkitt's lymphoma cells revealed that, while both lines responded to radiation with an enrichment of cells in the S and G_2/M phases of the cell cycle at 12 h after treatment, the BL30 cells clearly showed an apoptotic peak of fragmented DNA to the left of the G_1 peak at 12 h and 24 h post-irradiation. Whereas BL30 cells progressed into apoptosis accompanied by diminished G_1 and some accumulation in S-phase, the more resistant cell line, BL30(S), accumulated almost entirely in G_2/M by 24 h post-irradiation (Figure 2).

CDK activity in response to radiation

Since these two isogenic lines, exhibiting different amounts of apoptosis, behaved quite differently in their distribution through the cell cycle post-irradiation, it provided the opportunity to investigate the potential role of CDK activation in the process of apoptosis. Utilising precipitating antibodies to $p58^{cyclinA}$, the specific kinase activity of cyclin A was monitored with histone H1 substrate during radiation-induced apoptosis over a 12 h period in response to 10 Gy of radiation. A representative autoradiograph of $p58^{cyclinA}$ kinase activity is presented in Figure 3a. While activity was significantly inhibited at 4 h post-irradiation in both cell types (to approximately 35–40% of unirradiated values), activation of $p58^{cyclinA}$-associated kinase activity or recovery of this activity in BL30 cells to 110% of unirradiated values by 12 h contrasts with the persistent inhibition of kinase activity in the resistant cell line, BL30(S) (Figure 3b). This three-fold activation or recovery (between 4 h and 12 h) in BL30 cells coincides with the progression of a significant proportion of cells into apoptosis. Similar data were obtained when immunoprecipitation was carried out for CDK2 and $p33^{cdk2}$ kinase activity determined after 10 Gy or radiation (Figure 3c).

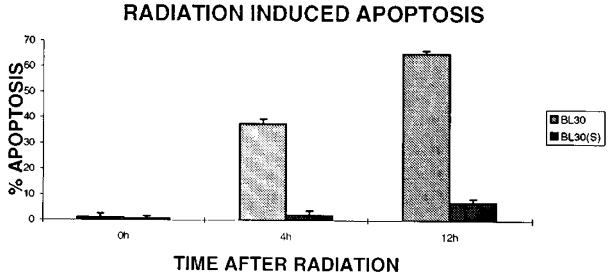

Figure 1 Radiation-induced apoptosis in Burkitt's Lymphoma cells. BL30 and BL30(S) cells grown to log-phase were exposed to 10 Gy of γ-irradiation. Samples of cells were removed just prior to irradiation 0 h, 4 h or 12 h after exposure, then fixed and stained wtih Hoechst 33258. Apoptosis was quantitated by counting the number of apoptotic cells, and expressing the proportion of cells undergoing apoptosis as a percentage of total. Duplicates of a representative experiment are presented and error bars represent SEM.

Regulation of CDK activity

Western blotting of cell lysates prepared from irradiated cells revealed that the cellular abundance of $p33^{cdk2}$ and $p58^{cyclinA}$ does not significantly change during apoptosis in the two cell types (Figure 4a and b). In addition, there was no significant difference between the two cell lines examined in the migration of $p33^{cdk2}$ suggesting that tyrosine phosphorylation was not involved in the differential regulation of kinase activity (Figure 4a).

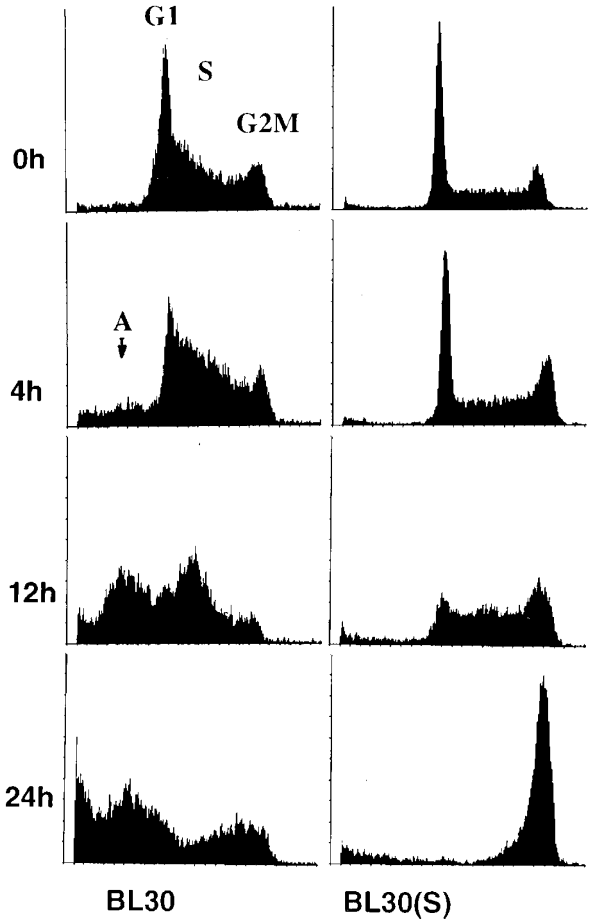

Figure 2 Cell cycle effects of radiation. Cells were irradiated as described in the legend to Figure 1, 10^5 cells were isolated at the indicated times, fixed in 50% ethanol at 4°C for 60 min, washed and resuspended in 0.5 ml of PBS containing RNAse (1 mg/ml) and then stained with 1 ml of propidium iodide (Sigma, 100 μg/ml in PBS). DNA profiles were obtained by FACS analysis on a Becton Dickinson FACScan utilising Lysis II software. DNA profiles indicate the relative abundance of G_1, S and G_2/M phase populations, as well as the emerging apoptotic peak of BL30 cells at 4 h and 12 h. A, denotes peak of apoptotic cells.

A signal transduction pathway operating through p53 and $p21^{WAF1}$, in response to radiation damage, has been demonstrated to lead to inhibition of CDK activity (El-Deiry *et al*, 1994, Canman *et al*, 1994, Khanna *et al*, 1995). Accordingly, we investigated changes in both p53 and $p21^{WAF1}$ after radiation exposure in these cells.

In agreement with previous findings (Khanna *et al*., 1996), p53 is induced significantly in BL30 cells, whereas BL30(S) cells have a constitutively elevated level of p53 expression which is poorly responsive to irradiation (results not shown). The expression of $p21^{WAF1}$ was also examined during the radiation response, and while expression in cellular lysates was relatively low compared to control lymphoblastoid cells, specific immunoprecipitation coupled with Western blotting for $p21^{WAF1}$, revealed an increase in $p21^{WAF1}$ at 4 h in both cell types (Figure 4c). Intriguingly, $p21^{WAF1}$ levels declined in BL30 cells by 12 h, revealing a pattern of expression which mirrored the inhibition in cyclin A-associated kinase activity (see Figure 3a). On the other hand $p21^{WAF1}$ continued to remain high at 12 h post-irradiation in the resistant cell line, BL30(S). In keeping with the transformed nature of these cells, $p21^{WAF1}$ immunoprecipitates were not associated with specific histone H1 kinase activity (results not shown).

These results suggest that the sustained increase in $p21^{WAF1}$ in BL30(S) cells leads to association with CDK complexes causing inhibition whereas in BL30 cells $p21^{WAF1}$ levels decrease with time post-irradiation and thus kinase activity is restored. This was checked by determining $p21^{WAF1}$ association with cyclinA–cdk2 kinase by immunoprecipitation with anti-cdk2 antibodies.

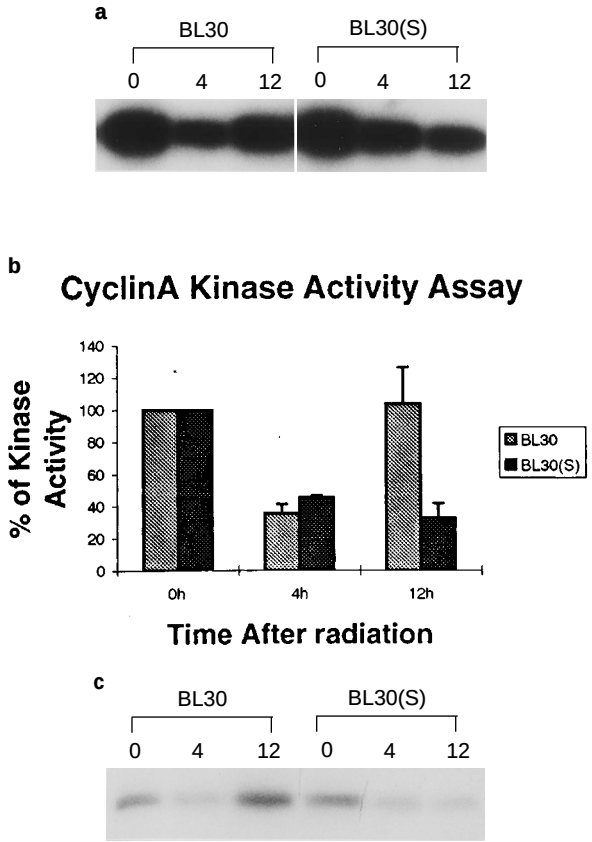

Figure 3 Activation of $p58^{cyclinA}$-associated kinase activity in apoptosis. (**a**) autoradiograph showing histone H1 kinase activity associated with $p58^{cyclinA}$ at 0, 4 and 12 h after irradiation (10 Gy). Kinase activity was determined by utilising a monoclonal antibody to specifically immunoprecipitate $p58^{cyclinA}$ complexes and assay associated histone H1 kinase activity. After completion of the kinase reaction, the reaction mixture was run on a 12% SDS–PAGE gel, and the dried gel was exposed to Kodak X-omat film. (**b**) Cyclin A kinase activity was quantitated using Image Quant Software on a Molecular Dynamics Phosphorimager, and is expressed as a percentage of control, unirradiated cells. Data are presented for at least three experiments and error bars represent SEM. (**c**) Autoradiograph showing histone H1 kinase activity associated with $p33^{cdk2}$ complexes at 0, 4 and 12 h after irradiation (10 Gy).

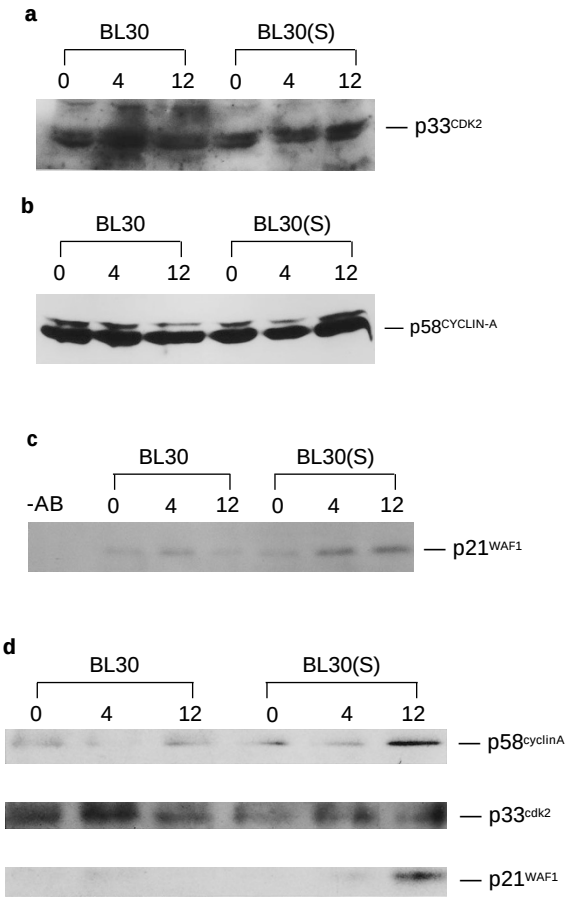

Figure 4 Expression of cell cycle regulators in apoptosis. Detergent lysates were prepared from BL30 and BL30(S) cells irradiated with 10 Gy, and incubated for the times indicated. Following SDS–PAGE on 12% gels, proteins were immunoblotted with (**a**) antibodies specific for $p33^{cdk2}$ (**b**) antibodies to $p58^{cyclinA}$ (**c**) Immunoprecipitation of $p21^{WAF1}$ coupled with Western blotting with anti-$p21^{WAF1}$ antibody (**d**) association of $p21^{WAF1}$ with cyclinA–Cdk2 complex. Coupled immunoprecipitation (antiCdk2) and Immunoblotting with antibodies to Cdk2, CyclinA and WAF1 was carried out on extracts prepared at 0, 4, 12 h after irradiation.

As expected no differences were noted in the relative abundance of $p58^{cyclinA}/p33^{cdk2}$ complexes (Figure 4d). The amount of $p21^{WAF1}$ associated with the cyclinA–cdk2 complexes increased with time in BL30(S) cells, compatible with the inhibition of CDK activity, whereas there was only a weak tansient increase in BL30 (Figure 4c).

Effect of radiation on $p21^{WAF1}$ stability

Since it was possible that the changes in $p21^{WAF1}$ might be due to turn-over rate, we determined the effect of radiation on stability of induced $p21^{WAF1}$ in both BL30 and BL30(S) cell lines using cycloheximide (CHX). BL30 and BL30(S) cells were treated with radiation and incubated for 4 h prior to addition of CHX and cell lysates were subsequently prepared at various time intervals and assayed by immunoblotting with $p21^{WAF1}$ antibodies. Under these conditions $p21^{WAF1}$ was totally degraded in the apoptosis-susceptible cell line BL30 post-irradiation but remained stable in BL30(S) the resistant cell line (Figure 5). Since cycloheximide has been shown to either inhibit or enhance apoptosis (Baxter and Lavin, 1992) we determined whether this compound might affect the rate or extent of apoptosis which might complicate the interpretation of the $p21^{WAF1}$ stability data. The results in Table 1 demonstrate the cycloheximide (25 μg/ml) had no effect on apoptosis in the two cell types.

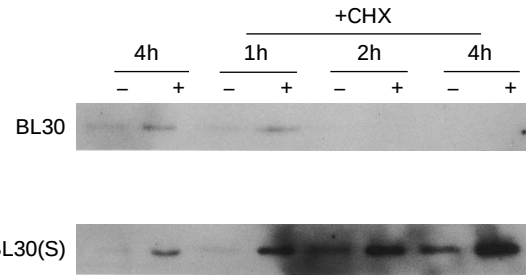

Figure 5 Effect of radiation on $p21^{WAF1}$ stability: Stability of radiation-induced $p21^{WAF1}$ in BL30 and BL30(S) cells was determined over a 4 h period after treatment with 10 Gy of radiation. Cells were lysed after 25 μg/ml cycloheximide (CHX) was added at the indicated times (1, 2, and 4 h). Total protein (20 μg) was analyzed by Western blotting as described in methods. –,+ symbols stand for minus or plus radiation, respectively.

Table 1 The effect of cycloheximide on BL30 and BL30(S) cells post-irradiation

Time	Cell lines			
	BL30	BL30+CHX	BL30(S)	BL30(S)+CHX
4 h	31%[a]	32%	2%	3%
5 h	40%	43%	3%	4%
6 h	50%	51%	5%	6%
8 h	70%	72%	10%	12%

[a]The percentage of apoptotic cells are means of data from 3–6 experiments.

Discussion

The potential role of activation of cyclin-dependent kinases in the initiation and/or the progression through apoptosis has recently been investigated in different cell types. While studies have implicated the activation of cyclin A-dependent kinases in chemically-induced apoptosis of S-phase Hela cells (Meikrantz *et al*, 1994) and in myc-overexpressing fibroblasts (Hoang *et al*, 1994), others have pointed to a role for selective activation of $p34^{cdc2}$-associated kinases in protease-induced apoptosis in lymphoma cells (Shi *et al*, 1994); cyclinB1/cdc2 kinase complexes in DNA damage-induced apoptosis (Shimizu *et al*, 1995) and cyclin E-, but not cyclin A-, containing complexes in cytosine arabinoside-induced apoptosis of HL60 cells (Dou *et al*, 1995). Meikrantz *et al* (1994) demonstrated that apoptosis can be induced in HeLa cells by the same agents that induce premature mitosis in hamster cells. Under these conditions cyclin A-dependent kinase activity increases. When L929 fibroblasts are arrested early in G_1 phase, prior to cyclin A synthesis they are protected from TNFα-induced apoptosis (Belizario and Dinarello, 1991). On the other hand when positive regulators of cyclin A transcription, c-*myc* and adenovirus E1A, are ectopically expressed they lead to apoptosis (Buchou *et al*, 1993, Jansen-Durr *et al*, 1993). Shi *et al* (1994) have suggested that the initiation of abortive mitosis through premature $p34^{cdc2}$ activation may be a general mechanism for induction of apoptosis, acting as a convergence point for different apoptotic signals. More recent data employing FT-210 cells exposed to a diverse array of apoptotic-inducing stimuli, where $p34^{cdc2}$ was degraded, failed to change susceptibility to undergo apoptosis indicating that $p34^{cdc2}$ activation was not generally obligatory for apoptosis (Martin *et al*, 1995). In addition Norbury *et al* (1994) have shown that activation of cdc2 is not involved in the induction of apoptosis in quiescent thymocytes. However, it is possible that other cyclin-dependent kinases are active in promoting apoptosis in non-proliferating cells.

The model system which we have employed in this study has the inherent advantage of utilising an isogenic pair of well characterised cell lines. The resistant cell line BL30(S) provides an excellent control for comparison of kinase activation associated with radiation-induced apoptosis. While both cell lines demonstrate inhibition of kinase activity at 4 h, BL30 specifically activates $p58^{cyclinA}$-dependent kinase activity as cells progress through apoptosis. However, the temporal relationship observed between apoptosis and kinase activation precludes a causal relationship from being established at this time. Clearly the trigger for apoptosis has been activated early in BL30 cells and an appreciable number of apoptotic cells are present at 4 h post-irradiation by which time CDK activity has reached a minimum in the two cell types. The increased stability of $p21^{WAF1}$ in the resistant line, BL30(S), may be a mechanism to prevent premature activation of CDK activity which might ultimately allow these cells to proceed into mitosis prior to repair of cellular damage. Such a role for $p21^{WAF1}$ is in agreement with recent data demonstrating that p21-mediated growth arrest can protect cells from apoptosis induced by p53 (Polyak *et al*, 1996). In the case of BL30 once the process is triggered stabilisation

of $p21^{WAF1}$ would not be required. Indeed, while apparently conflicting reports describing the role of activation of specific cyclin-dependent kinase(s) in apoptosis continue to emerge we have observed that both cell lines described here are highly susceptible to wortmannin-induced apoptosis independent of cyclin-dependent kinase activation, suggesting that kinase activation *per se* is not part of a universal mechanism for programmed cell death.

The parent cell line BL30 is mutant for p53 in its remaining allele (Farrell *et al*, 1991), and responds to radiation with a significant induction of p53 within 4 h, which persists for at least another 8 h (data not shown), in contrast to the response of BL30(S) in which the elevated basal level of p53 is only slightly increased in response to radiation (Khanna *et al*, 1996). However, the BL30 cells significantly induce $p21^{WAF1}$ in response to irradiation at 4 h and while p53 remains significantly elevated, $p21^{WAF1}$ expression is significantly reduced by the 12 h point, suggesting that $p21^{WAF1}$ (in)stability may play some role in regulation of cyclin-dependent kinase activity and G_1 arrest (Bae *et al*, 1995). Determination of $p21^{WAF1}$ protein stability demonstrated that this was the case, with the protein being considerably more stable in the resistant cell line. An inverse correlation is noted between $p58^{cyclinA}$-dependent kinase activity and $p21^{WAF1}$ expression.

The observed lack of associated G_1 arrest in either cell line is consistent with previous studies which implicate the necessity of a functional p53 in mediating G_1 arrest (El-Deiry *et al*, 1994, Bae *et al*, 1995). Recently it has been established that p21 deficient cells are unable to arrest in G_1 following irradiation, yet loss of p21 in intestinal epithelial cells does not prevent p53-dependent apoptosis in response to irradiation (Brugarolos *et al*, 1995). In addition in p53 deficient cells, radiation-induced apoptosis can occur in association with a G_2/M arrest (Han *et al*, 1995, Allday *et al*, 1995).

In summary we have shown that CDK activity is reduced in a pair of isogenic Burkitt's lymphoma cells in response to ionizing radiation. The recovery of CDK activity to normal levels is associated with a loss of $p21^{WAF1}$ protein and the progress of apoptosis in the susceptible member of the pair, BL30. On the other hand the resistant cell line (BL30(S)) is characterised by a continued inhibition of CDK activity. The results obtained here for BL30 cells are somewhat different from other cells undergoing apoptosis where activation of CDK activity was observed (Meikrantz *et al*, 1994, Dou *et al*, 1995). In this study a decrease in CDK activity was initially observed followed by recovery of full activity with time. We have not been able to distinguish between a reversal of inhibition of CDK activity in all cells in the irradiated population and the activation of CDK activity in a sub-population, possibly associated with the onset or progress of apoptosis.

Materials and Methods

Cell culture

The two isogenic cell lines established from an EBV-negative Burkitt's lymphoma biopsy, BL30, had either retained the original group I phenotype (BL30 parent) or had progressed to a group II/III phenotype (BL30(S)). Utilising highly polymorphic microsatellite markers, it has been established that these two cell lines are genotypically identical; full characterisation of these cell lines appears elsewhere (Khanna *et al*, 1996). The cells were routinely maintained in exponential growth in RPMI 1640 medium containing 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and foetal calf serum at 10% (BL30(S)) or 20% (BL30).

Detection of radiation-induced apoptosis

BL30 and BL30(S) cells were exposed to 10 Gy of γ-rays from a ^{137}Cs source (3 Gy/min) and apoptosis was determined over a 12 h period. At the end of each incubation, cells (10^6) were fixed with two volumes of Carnoy's fixative, washed by CPB buffer, and stained with 10 nM Hoechst 33258. Apoptotic cells were counted using a Zeiss Axioskop fluorescence microscope. One hundred cells were counted, and the percent apoptosis represents the average of three independent experiments.

Flow cytometry

DNA content analysis was according to an established protocol (Nicoletti *et al*, 1991). Briefly, 10^6 cells were fixed in 2 ml of cold 70% ethanol for 60 min. Following washing in PBS, the cell pellet was resuspended in 0.5 ml of PBS to which 0.5 ml of 1 mg/ml RNAse was added. Propidium Iodide (1 ml of 100 μg/ml) was gently mixed and the cells were incubated in the dark at room temperature for 15 min. Analysis was performed on a Becton-Dickinson FACScan utilising Lysis II software.

Immunoblotting and immunoprecipitation

Cell lysates were prepared from logphase cells utilising a previously characterised lysis buffer (Williams *et al*, 1993). Immunoprecipitations and subsequent kinase reactions were performed as previously described (Williams *et al*, 1993), utilising histone H1 as a substrate and anti-cdk2 (2 μg of rabbit IgG), anti-cyclin A (100 μl of cell culture supernatant of mouse monoclonal (AMAb 5-39), and anti-p21 (2 μl of rabbit sera) antibodies and 250 μg (kinase reaction) or 1.0 mg (immunoprecipitations) of pre-cleared cell lysate. Quantitation of the kinase activity was performed by exposure of the dried histone H1 gels to a Phosphorimager plate and analysis with a Molecular Dynamics Phosphorimager and Imagequant software.

Western blotting was performed as described previously (Williams *et al*, 1993), with the exception that electrophoretic transfer was accomplished in a 10 mM CAPS buffer pH 11.0, 10% methanol for 2 h at 50 V. Primary antibody was diluted to 1:1000 (CDK2) or 1:100 (WAF1 monoclonal antibody, Oncogene Science) in 5% Skim milk in PBS-0.05% Tween 20 (PBS-T) or used undiluted (cyclin A monoclonal), and incubated with pre-blocked membranes for 1 h at room temperature. Washing and secondary antibody (1 : 2000 dilution of horseradish peroxidase conjugated goat anti-rabbit, or anti-mouse antibodies, Sigma) were followed by extensive washing in PBS-T, and developed using an ECL based detection procedure according to the Manufacturer (DuPont NEN).

Coupled immunoblotting

Cell lysates were prepared and 1 mg of precleared protein extracts was incubated with 2 μg of Cdk2 antibody overnight at 4°C. The antibody complex was captured by the addition of 50 μl of Protein A agarose for 30 min at 4°C, pelleted by centrifugation at 400 g for

1 min, and washed three times with 1 ml each of lysis buffer. Samples were boiled in sample loading buffer for 5 min and then electrophoresed on 15% SDS-polyacrylamide gels. The proteins were immunoblotted as described previously and screened with Cdk2 (1:1000 dilution). The same Western blots were stripped by heating to 50°C in buffer (2.5 mM Tris, pH 6.8, 1 mM β-mercaptoethanol, 2% SDS) for 30 min. The filters were then washed with copious amounts for PBS-Tween and rescreened with WAF1 (Oncogene Science Inc.), and cyclin A antibodies, the resulting ECL blots were overlaid to ensure the correct positioning of the bands.

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