ANTICANCER RESEARCH *25*: 1123-1130 (2005)

The Expression of p21/WAF-1 and Cyclin B1 Mediate Mitotic Delay in x-Irradiated Fibroblasts

MICKAEL J. CARIVEAU[1,2], CHARLES J. KOVACS[2], RON R. ALLISON[2], ROBERTA M. JOHNKE[2], GERHARD W. KALMUS[3] and MARK EVANS[2]

[1]*Department of Physics, East Carolina University, Greenville NC, 27858;*
[2]*Department of Radiation Oncology, The Brody School of Medicine, East Carolina University, Greenville NC, 27858;*
[3]*Department of Biology, East Carolina University, Greenville NC, 27858, U.S.A.*

Abstract. *Background: To better understand the relationship between mitotic delay and the disruption of cyclin B1 and p21 in x-irradiated fibroblasts, studies were carried out to establish correlations between the downregulation of cyclin B1 by the cyclin kinase inhibitor (CKI) p21 and the induction of mitotic delay in the NIH3T3 fibroblast. Materials and Methods: Cell cycle kinetics were used to analyze mitotic delay in irradiated NIH3T3 cells and immunocytochemistry incorporated to assess the expression of cyclin B1 and p21, following 2 or 4Gy x-irradiation. Results and Discussion: Results indicate a dose dependent increase in mitotic delay accompanied by a downregulation of cyclin B1 and corresponding upregulation of the CKI p21 in exponentially growing cultures. Data indicates that the induction of radiation – induced division delay appears to be dependent on the p21 inhibition of cyclin B1 and, furthermore, p21 and cyclin B1 expression are highly dependent on cell density.*

Recently, a significant correlation has been shown to exist between residual DNA damage and the development of late radiation fibrosis, a chronic, progressive, and untreatable disease (1, 2). This condition, at the cellular level, has been attributed to the abnormal proliferation of fibroblasts. However, the cellular/molecular mechanisms triggering normal fibroblasts to undergo fibrotic changes in cancer survivors have not been identified. Furthermore, it has been suggested, but not proven, that these lesions are a direct result of an irreversible cell cycle arrest (3-5).

Correspondence to: Mickael J. Cariveau, Ed Warren Life Sciences Building, RM013, Division of Radiation Biology and Oncology, ECU Brody School of Medicine, Greenville, NC 27858, U.S.A. Fax: 252-744-3775, e-mail: mjc0302@mail.ecu.edu

Key Words: DSB, mitotic delay, signaling proteins, cell cycle analysis.

Initiation and maintenance of cell cycle arrest is regulated by a group of proteins known as the cyclins which function by coupling to their corresponding cyclin dependent kinases (CDK) (6-9). Of particular interest to cell cycle arrest in response to DNA damage is the G_2/M damage checkpoint which is regulated by cyclin B1/CDK1 and initiated by DNA damage activation of the cyclin kinase inhibitor p21(10-14). The inhibitory potential of p21 is well established; however, there is still much confusion about the role of p21 in the irradiated cell (15, 16). While several studies have investigated the role of p21 – cylcin B1 interactions in the irradiated cell, most of these investigations have been performed in tumor cell lines or confluent cell cultures which are known to exhibit abrogated cell cycle machinery (17-24). To more fully understand the generation of fibrotic lesions in normal irradiated tissues, we have chosen as the model the NIH3T3 fibroblasts which express functional cell cycle regulatory proteins. The results indicate a density – dependent expression of p21 and cyclin B1, and a compelling correlation between radiation – induced division delay and the simultaneous up/down regulation of the CKI p21/cyclin B1 following 2 or 4Gy x-irradiation.

Materials and Methods

Cell cultures, cell kinetic analysis, and flow cytometry. Unless otherwise mentioned, NIH3T3 murine fibroblasts (ATCC # - CRL-1658, passage #200) were maintained in exponential growth at 75-85% confluency in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% bovine calf serum (BCS) at 37˚C in 5% CO_2 – 95% humidified air. For cell kinetic analysis, exponentially-growing NIH3T3 cells were harvested, seeded into 6-well plates containing sterile 22cm^2 coverslips, incubated for 24 hours, and then irradiated (from 1-6Gy). Samples were harvested over a 24 hour period following irradiation, washed in PBS and fixed in –20˚C methanol, allowed to dry, attached to slides and DNA stained with the Schiff reaction. The mitotic index was established as the number of mitotic figures scored out of 1000 cells.

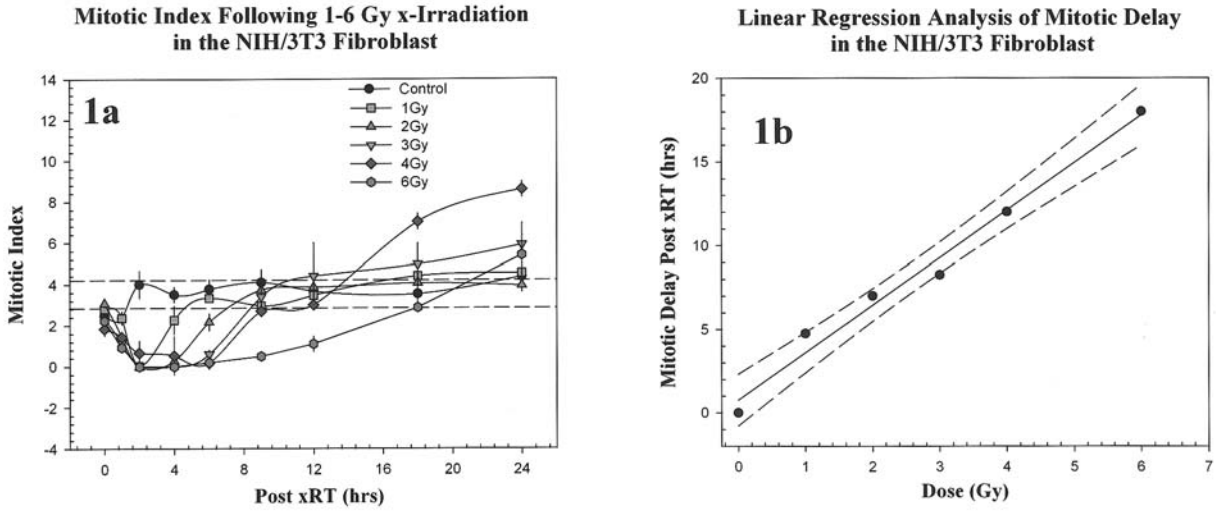


Figure 1a and 1b. *Radiation induced division delay is shown in figure 1a as the total number of mitotic figures for dose ranges from 1-6Gy. Each data point represents the number of mitotic figures from a population of 1000 cells from 3 independent experiments with the dashed lines representing the standard deviation of the control (–●–). 1b shows a linear regression analysis of the radiation-induced mitotic delay as determined from 1a where the slope yields a mitotic delay of 2.65min/cGy.*

For flow cytometric analysis of mitotic delay, exponentially growing cultures were irradiated (4Gy) and then incubated from 0.25-24 hours. At indicated times, cells were harvested, resuspended to 1 x 10^6 cells/ml, and fixed in –20˚C methanol. On the day of analysis, cells were treated with ribonuclease A (RNase A, 1mg/ml) (Sigma, R5500) for 30 minutes at 37˚C, then stained with propidium iodide (PI, 20mg/ml) (Sigma, P1470). Stained nuclei were analyzed for PI fluorescence using a Beckton Dickson FACScan flow cytometer, and data analyzed by ModFit 2 (Verity Software House Inc., Topsham, ME). The proportion of cells in G_0/G_1, S, and G_2/M phases of the cell cycle were determined from a minimum of 10,000 events acquired at ≤400 events per second.

Cell density and radiation induced expression of cyclin B1 and p21. To study density dependent expression of p21 and cyclin B1, exponentially growing NIH3T3 cells were plated into 35mm dishes containing a 22 cm^2 coverslips at densities from 5x103 - 6.0x10^4 per coverslip and incubated for 24 hours prior to washing in PBS and fixation in –20˚C methanol. For irradiation studies, 5x10^3 were grown as described above, irradiated (2 or 4Gy) and harvested from 0.25 – 24 hours respectively. Immunohistochemistry was then incorporated to determine the temporal expression of cyclin B1 and p21 following x-irradiation (2 or 4Gy). Briefly, fixed cells were permeabilized in Tris-buffered saline (TBS)/0.1% Triton X-100, blocked with 1.5% goat serum in PBS for 1 hour, 100ml of primary antibody (titrated for minimal background), either rabbit polyclonal p21 (Santa Cruz Biotrech., sc-756, 2mg/ml) or cyclin B1 (Santa Cruz Biotech., sc-752, 4mg/ml), diluted in blocking serum, was added, and cells incubated overnight at 4˚C. Cells were washed in TBS, treated with biotinylated goat anti-rabbit IgG (Santa Cruz, sc-2040, 5mg/ml) for 1 hour, rinsed twice in TBS, incubated in ABC reagent (Vector, PK-4000), and resolved with DAB (Vector, SK-4100). Samples were counterstained with Eosin and four samples from 3 independent experiments for a total of 12 counts per data point were then scored. The number of cells expressing nuclear p21 or cytoplasmic cyclin B1 was determined from a total of 500 cells. The mean number of positive cells from 3 independent experiments were used to determine data points and statistical significance was set at $p \leq 0.05$.

Results

Cell cycle analysis and growth parameters. Analysis of radiation-induced mitotic delay was investigated using traditional DNA labeling and flow cytometry to determine cell cycle kinetics. As shown in Figure 1a, irradiation of NIH3T3 cells leads to a dose dependent increase in division delay. The dashed lines in the figure represent the control ±1 standard deviation, and the point at which the experimental curves cross the lower control line indicates the mitotic delay in hours for that radiation dose. As shown, the delay is dose-dependent, rising from 5 hours at 1Gy to 18 hours at 6 Gy. Linear regression analysis projected a mitotic delay of 2.65min/cGy (Figure 1b).

To better understand cell cycle kinetics, cells were irradiated to a total dose of 4Gy and harvested over 18 hours for flow cytometric analysis. DNA histograms modeled with ModFit software are depicted in Figure 2. Figure 2a represents a DNA histogram of the control population with G_o/G_1 comprising 43.34% of the population, 45.21% making up S, and the remainder (11.45%) in G_2/M. At 1 hour post – irradiation, there is no change in cell cycle distribution (Figure 2b-d), but, within 2

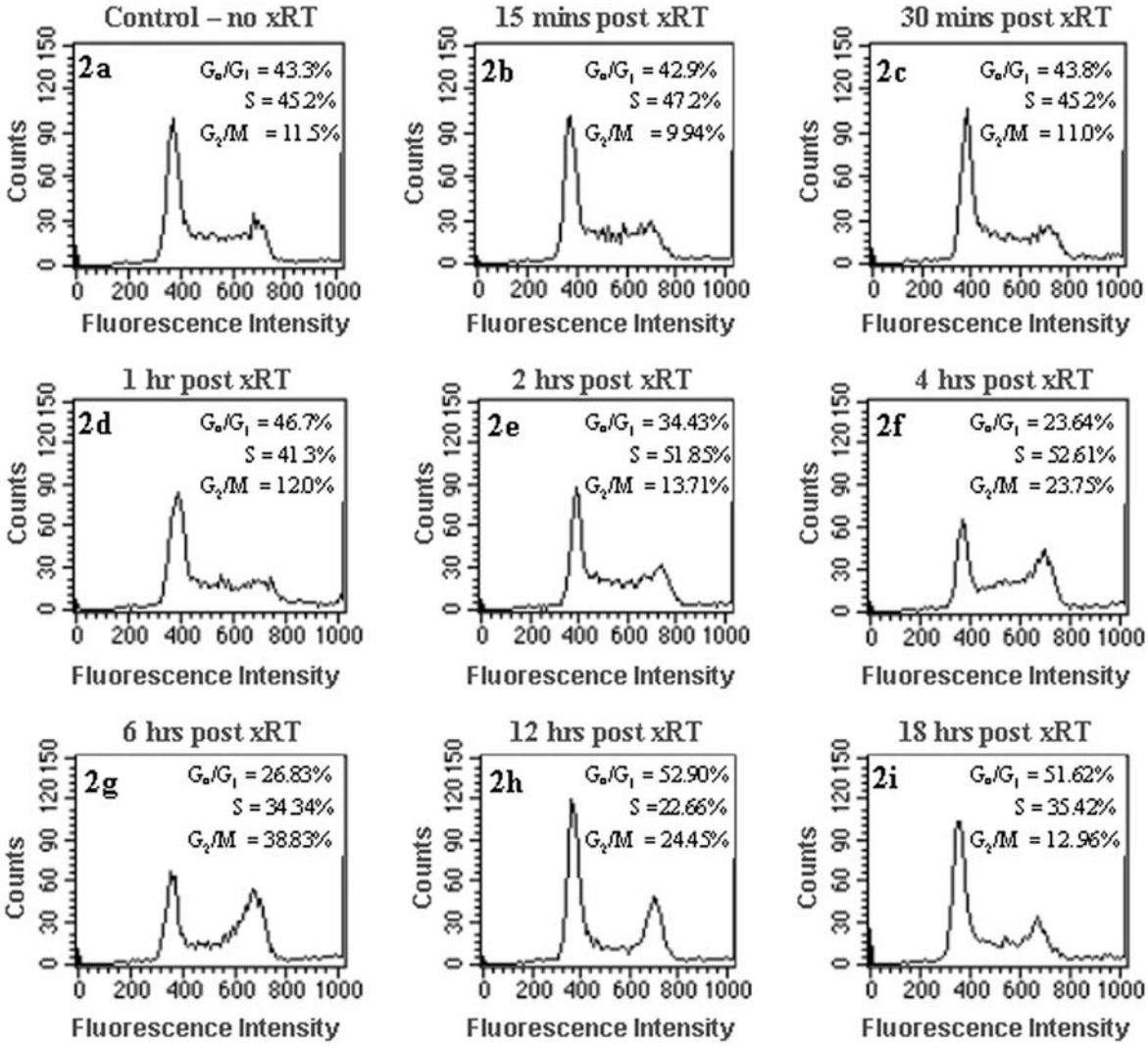


Figure 2a – i. *FACS cell cycle analysis. The representative images show the movement of cells through the cell cycle following exposure to a dose of 4Gy x-irradiation. Figure 2a represents the control, just prior to irradiation while 2b – 2i represent 0.25 – 18hrs post irradiation.*

hours, a pile up of cells within S phase is apparent (Figure 2e). The S – phase pile up remains at 4 hours (Figure 2f), while the G_2/M population has increased to 23.75%. Cells remain blocked at G_2/M through 6 hours with a maximal population of 38.83% (Figure 2g). By 12 hours, the cell cycle block is diminishing (Figure 2h), and by 18 hours, the population has redistributed to pre-irradiated control levels (Figure 2i). The data obtained from flow cytometry correlates strongly with that obtained from DNA labeling studies where cell proliferation remains suppressed from 1-6 hours with a slow recovery occurring from 6 - 12 hours and a return to preirradiation distributions by 18 hours.

Effects of cell density on the expression of cyclin B1 and p21. The NIH3T3 fibroblast is highly contact inhibited, therefore, to ensure that cyclin B1 and p21 expression occurred in response to xRT and not contact inhibition, exponentially growing cultures were plated on sterile 22 cm^2 coverslips ($5x10^3$ – $6x10^4$ or 58-700 cells/mm^2), incubated for 36 hours, and then analyzed for expression of p21 and cyclin B1 *via* immunocytochemistry. Data indicates that cyclin B1 expression decreases while p21 expression increases in relation to increasing cell density, where $5x10^3$ cells per dish produced minimal background staining (data not shown).

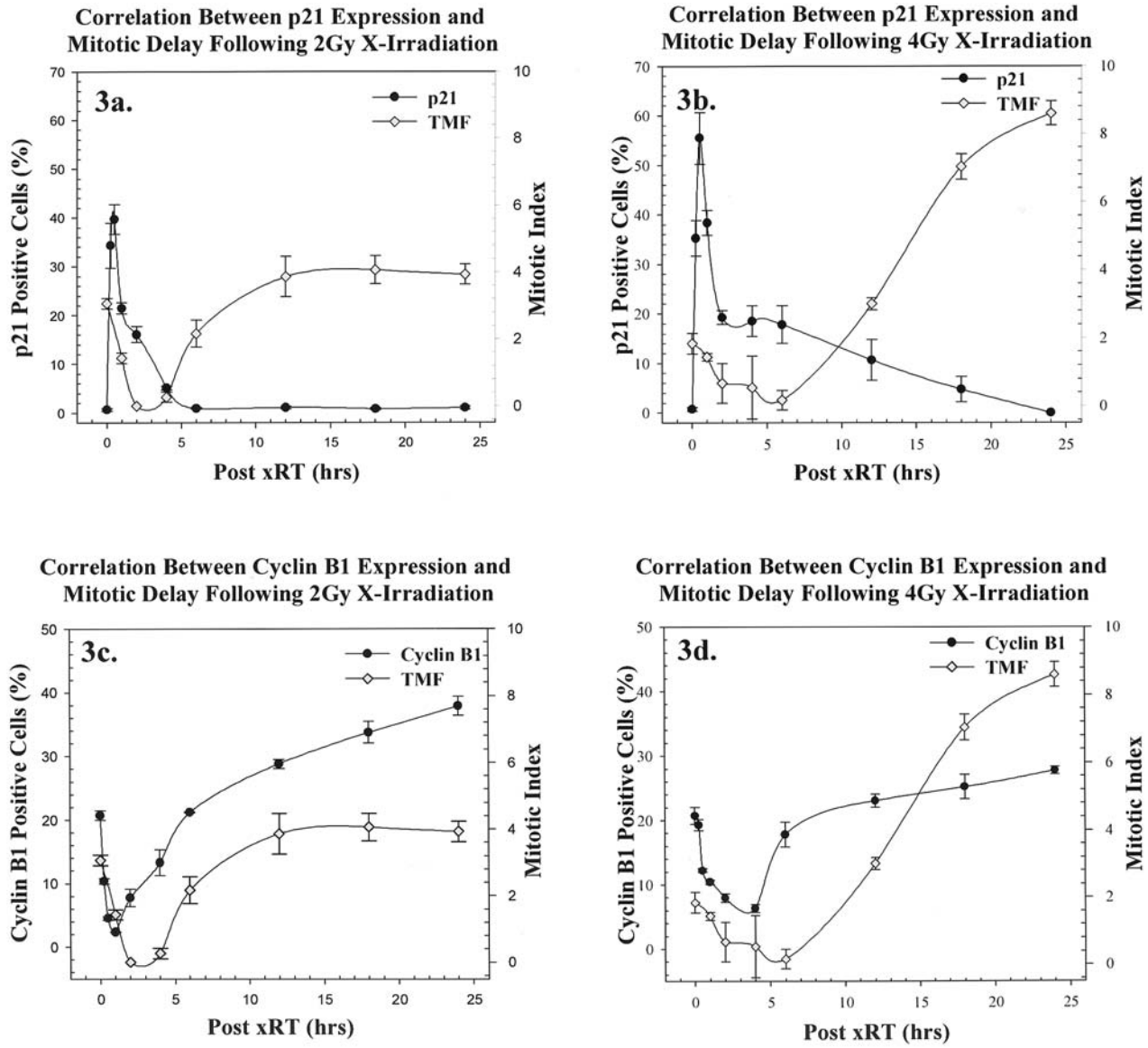
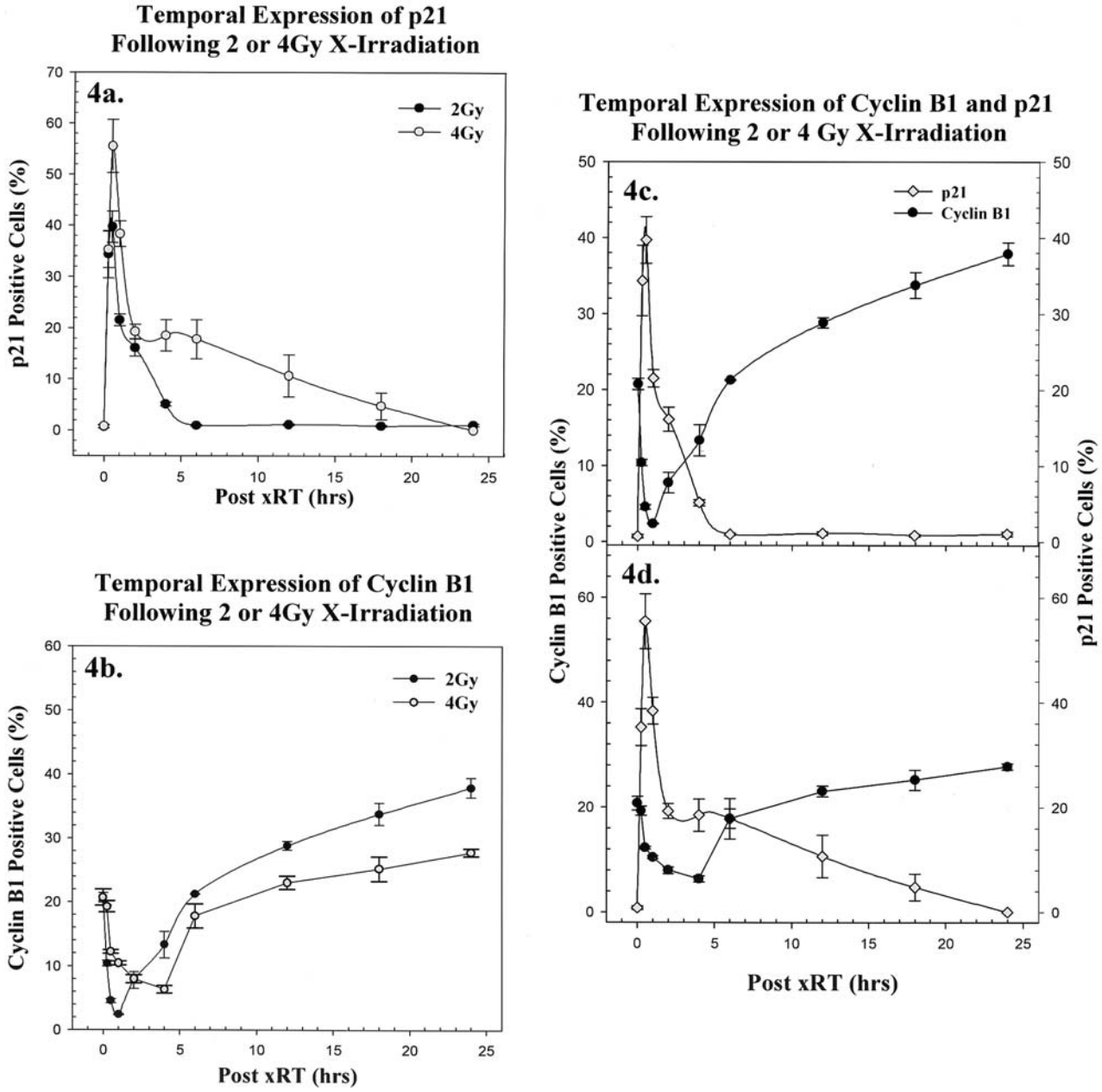


Figure 3a – d. *Temporal expression of cyclin B1 and p21 after 2 or 4Gy x – irradiation. Figures 3a and 3b show the temporal expression of p21 following 2 or 4Gy xRT and its relation to mitotic delay. The temporal expression of cyclin B1 following 2 or 4Gy x-irradiation is shown in 3c and 3d. Each data point represents the mean of 3 independent experiments and a population of 500 cells with the error bars as the standard deviation of the mean.*

Temporal expression of cyclin B1 and p21 following x-irradiation. Figure 3 describes the temporal expression of cyclin B1 and p21 in the x-irradiated cell and its correlation to mitotic delay based on the mitotic index. Figure 3a describes the correlation of p21 activity with mitotic delay at a dose of 2Gy. As shown, p21 activity reaches a maximum of 41% rapidly after irradiation (within 30 minutes), with mitotic delay being maximal 2 hours after. Levels of p21 activity remain elevated for 2 hours before returning to preirradiated levels by 6 hours. In 3b, a dose increase to 4Gy leads to a greater percentage of cells expressing p21 (55% within 30 minutes) with levels returning to control distributions by 24 hours. Figure 4a depicts the difference in p21 expression at 2Gy and 4Gy, where a similar trend occurs within both groups with the magnitude and duration of expression greater at 4Gy, and the return to preirradiation control levels significantly delayed.

Cyclin B1 levels demonstrate a dose – dependent decrease in activity following exposure to x-irradiation. Following 2Gy x-irradiation (Figure 3c), cyclin B1 activity



Figures 4a – d. *Represent the relationship between the temporal expression of p21 and cyclin B1 following 2 or 4Gy x-irradiation. Each data point represents the mean of 3 independent experiments and a population of 500 cells with the error bars as the standard deviation of the mean.*

decreases to a minimum within 1 hour, while mitotic delay reaches a minimum within 2 hours followed by a slow recovery over 10 hours. After 4Gy x-irradiation (Figure 3d), both cyclin B1 activity and mitotic delay display a similar trend, with maximal decreases being observed later (4 and 6 hours, respectively). Figure 4b shows a comparison between this response of cyclin B1 inhibition in cells exposed to 2 or 4Gy x-irradiation. Results demonstrate that the temporal inhibition of cyclin B1 following x-irradiation is immediate and dose dependent, lasting 1 or 4 hours, respectively.

The temporal expression of cyclin B1 as compared with p21 is shown in Figures 4c and 4d where increased p21 activity corresponds with a concomitant decrease in cyclin B1 activity within the first hour after irradiation (Figure 4c). With 2 hours, levels of p21 activity have fallen below 20%

and cyclin B1 activity has increased to 8% with control levels attained by 6 hours (Figure 4c). Figure 4d displays correlations between p21 and cyclin B1 activity following 4Gy x-irradiation where the trends are similar to that of 2Gy with maximal levels of p21 activity occurring within 30 minutes post-irradiation and minimal levels of cyclin B1 activity being observed by 4 hours.

Discussion

Although the radioresponse of cell cycle proteins have been studied, most of the literature has concentrated on supralethal doses of radiation (8 – 12Gy) performed in cell lines which may or may not have had normal, well functioning cell cycle machinery . These conditions may have contributed to some of the confusion which exists regarding the relationship between radiation-induced mitotic delay and p21 activity (25). For example, some studies have shown that p21 invokes a resistance to radiation-induced damage in tumor cells while others suggest it may, in fact, lead to enhanced radiosensitivity (26-30). In addition, it has recently been shown that TP53 and p21 are necessary for initiation and maintenance of G2/M delay following DNA damage involving the direct binding of p21 to cyclin B1/CDK1 complexes (31-34). The major goal of the studies therefore, was to develop a cell model known to possess normal, functional cell cycle proteins and to investigate the effect of sublethal, rather than supralethal, radiation doses (1 – 6Gy) on mitotic delay. Specifically, the proteins p21 and cyclin B1 which regulate cell entry into mitosis were investigated. Results demonstrated that sublethal radiation doses initiated a dose-dependent block in cell cycle progression, varying proportionally with increasing dose, and becoming well pronounced within 30 minutes of radiation exposure. Further investigation of this block using flow cytometry demonstrated that it was characterized by an initial G1/S delay followed by a sustained G2/M block up to 12 hours post irradiation.

Both the magnitude of response and temporal pattern of expression of p21 was dose dependent suggesting an important role in the initiation of mitotic delay (and, therefore, enhanced DNA damage repair) in cells exposed to 2 or 4Gy. This is supported by recent data which shows that p21 rapidly co-localizes at the site of the DNA double strand break within minutes after damage, explaining the rapid appearance of p21 seen in our experiments (30).

In addition, the reduction in cyclin B1 positive cells occurs simultaneously with the initiation of mitotic delay and p21 upregulation, where cyclin B1 expression, similar to mitotic delay, is dose dependent, decreasing to a minimum within 1 or 4 hours at 2 and 4Gy, respectively. This reduction in cyclin B1 protein levels occurs simultaneously with an increase in p21 levels (Figure 3c-d, 4c-d). The fact that mitotic delay recovers slightly faster than the reappearance of cyclin B1 positive cells may result from release of arrested cells in other stages of the cell cycle during the time of irradiation. It is well known that cyclin B1 plays a role in radiation-induced division delay, an interaction that has been shown to occur via the TP53-p21 mediated stress response (32). The data presented here substantiate and clarify the role of cyclin B1 in radiation-induced mitotic delay in a normal fibroblast.

Collectively, our data have shown that the induction of radiation – induced division delay appears to be dependent upon the activation of p21, which downregulates the G_2/M protein cyclin B1. Based on these findings, we have defined a model useful for the investigation of cell cycle regulation following irradiation of cells with intact cell cycle machinery at a dose range of x-irradiation in which mitotic delay is the predominant response to injury.

Acknowledgements

This publication is dedicated to the life and memory of Dr. C.J. Kovacs, mentor and friend, whom passed away during preparation of this manuscript.

References

1 Dickson J, Magee B, Stewart A and West C: Relationship between, residual radiation-induced DNA double strand breaks in cultured fibroblasts and late radiation reactions: a comparison of training and validation cohorts of breast cancer patients, Radiotherapy Onc *62*: 321-326, 2002.

2 Denham J and Hauer-Jensen M: The radiotherapeutic injury – a complex wound, Radiotherap Oncol *63:* 129-145, 2002.

3 Syljuasen RG, Krolewski, Little J: Molecular events in radiation transformation, Rad Res *155:* 215-221, 2001.

4 Rodemann H P and Bamberg M: Cellular basis of radiation – induced fibrosis, Radiotherap Oncol *35:* 83-90, 1995.

5 Hill R, Rodemann, Henry J, Roberts S and M. Anscher: Normal tissue radiobiology: From the laboratory to the clinic. Int J Rad Oncol Biol Phys *49:* 353-365, 2001.

6 Abraham R: Cell cycle checkpoint signaling through the ATM and ATR kinases. Gene Devel *15:* 2177-2196, 2001.

7 Qin J and Lei L: Molecular anatomy of the DNA damage and replication checkpoints. Rad Res *159:* 139-148, 2003.

8 Bartek J and Lukas J: Mammalian G1- and S-phase checkpoints in response to DNA damage. Curr Opin Cell Biol *13:* 738-747, 2001.

9 Morgan D: Cyclin-dependent kinases: Engines, clocks, and microprocessors. Annu Rev Dev Biol *13:* 261-291, 1997.

10 Porter L, Singh G and Lee J: Abundance of cyclin B1 regulates γ-radiation –induced apoptosis. Blood *95* 2645-2650, 2000.

11 Kao G, McKenna G, Maity A, Blank K and Muschel R: Cyclin B1 is a rate limiting component of the radiation induced G2 delay in HeLa cells. Canc Res *57:* 753-758, 1997.

12 Toyoshima F, Moriguchi T, Wada A, Fukuda M and Nishida E: Nuclear export of cyclin B1 and its possible role in the DNA damage-induced G_2 checkpoint. EMBO Jour *17*: 2728-2735, 1998.

13 Jin P, Hardy S and Morgan D: Nuclear localization of cyclin B1 controls mitotic entry after DNA damage. Jour Cell Biol *141:* 875-885, 1998.
14 Takizawa C, Weiss K and Morgan D: Ran-independent nuclear import of cyclin B1-cdc2 by importin b. Proc Natl Acad Sci USA *96:* 7938-7943, 1999.
15 LaBaer J, Garret M, Stevenson L, Slingerland J, Sandhu C, Chou H, Fattaey A and Harlow E: New functional activities for the p21 family of CDK inhibitors. Genes Dev *11:* 847-862, 1997.
16 HarperJ, Elledge S, Keyomarsi K, Dynlacht B, Tsai L, Zhang P, Dobrowolski S, Bai C, Connell-Crowley L, Swindell E, Fox M and Wei N: Inhibition of cyclin dependent kinases by p21. Mol Biol Cell *6*: 387-400, 1995.
17 Meerson A, Milyavsky M and Rotter V: p53 mediates density dependent growth arrest. Febs Lett *559*: 152-158, 2004.
18 Datto M, Li Y, Panus J, Howe D, Xiong Y and Wang X: Transforming growth factor b induces the cyclin – dependent kinase inhibitor p21 through a p53 – independent mechanism. Proc Natl Acad Sci USA *92:* 5545-5549, 1995.
19 Barker S, Eichele G, Zhang P, Rawls A, Sands A, Bradley A, Nolson E, Harper J and Elledge S: p53-independent expression of p21cip1 in muscle and other terminally differentiating cells. Science *267:* 1024-1027, 1995.
20 Stein G and Dulic V: Molecular mechanisms for the senescent cell cycle arrest. J Investig Dermatol Symp Proc *3:* 14-18, 1998.
21 Vjekoslav D,Drullinger L,Lees E, Reed S and Stein G: Altered regulation of G1 cyclins in senescent human diploid fibroblasts: Accumulation of inactive cyclin E-cdk2 and cyclin D1-cdk2 complexes. Proc Natl Acad Sci USA *90:* 11034-11038, 1993.
22 Ritt M, Mayor J, Wojcieszyn J, Smith III R, Barton C and Modiano J: Sustained nuclear localization of p21/WAF-1 upon growth arrest induced by contact inhibition. Cancer Lett *158*: 73-84, 2000.
23 Akimoto T, Seong J, Hunter N, Buchmiller L, Mason K and Milas L: Association of increased radiocurability of murine carcinomas with low constitutive expression of p21WAF1/CIP1 protein. Int J Radiation Oncology Biol Phys *44:* 413-419, 1999.
24 Bunz F, Dutrizux A, Lengauer C, Waldman T, Zhou S, Brown J, Sedivy J, Kinzler K and Vogelstein B: Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science *282:* 1497-1501, 1998.
25 Gartel A and Tyner A: The role of the cyclin dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther *1:* 639-649, 2002.
26 Dulic V, Stein G, Far D and Reed S: Nuclear accumulation of $p21^{Cip1}$ at the onset of mitosis: a role at the G2/M-phase transition. Mol Cell Biol *18:* 546-557, 1998.
27 Akimoto T, Seong J, Hunter N, Buchmiller L, Mason K and Milas L: Association of increased radiocurability of murine carcinomas with low constitutive expression of $p21^{WAF1/CIP1}$ protein. Int J Radiation Oncology Biol Phys *44:* 413-419, 1999.
28 Adimoolan S, Lin C and Ford J: The p52-regulated cyclin-dependent kinase inhibitor, p21 (cip1, waf1, sdi1), is not required for global genomic and transcription-coupled nucleotide excision repair of UV-induced DNA photproducts. J Biol Chem *276:* 25813-25822, 2001.
29 Cooper M, Balajee A and Bohr V: The C-terminal domain of p21 inhibits nucleotide excision repair *in vitro* and *in vivo*. Mol Cell Biol *10:* 2119-2229, 1999.
30 Jakob B, Scholz M and Taucher-Scholz G: Immediate localized CDKN1A (p21) radiation response after DNA damage produced by heavy-ion tracks. Rad Res *154:* 398-405, 2000.
31 Daino K, Ichimura S and Nenoi M: Early induction of CDKN1A (p21) and GADD45 mRNA by a low dose of ionizing radiation is due to their dose-dependent post-transcriptional regulation. Rad Res *157:* 478-482, 2002.
32 Innocente S, Abrahamson J, Cogswell J and Lee J: p53 regulates a G2 checkpoint through cyclin B1. Proc Natl Acad Sci USA *96:* 2147-2152, 1999.
33 Flatt P, Tang L, Scatena C, Szak S and Pietenpol J: p53 regulation of G2 checkpoint is retinoblastoma protein dependent. Mol Cell Biol *20:* 4210-4223, 2000.
34 De Souza C, Ellem K and Gabrielli B: Centrosomal and cytoplasmic Cdc2/Cyclin B1 activation precedes nuclear mitotic events. Exp Cell Res *257:* 11-21, 2000.

Received December 20, 2004
Accepted February 7, 2005