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Cyclin B1 expression in response to abrogation of the radiation-induced G_2/M block in HeLa cells

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Abstract. The G_2 block is a major response of cells to DNA damage and seem to be induced independently of p53 status. It is thought that the G_2 block has a protective function and allows cells to repair their DNA. The molecular events involved in the formation of the G_2 block therefore are of great interest. We have used pentoxifylline, a potent G_2 delay abrogator, to study the expression of an essential component of the mitosis promoting complex (MPF), cyclin B1. Cyclin B1/G_2 ratios are used to show that irradiation induces a decrease in cyclin B1 expression and that pentoxifylline restores cyclin B1 expression to control level. This confirms that suppression of cyclin B1 plays a role in the formation of the G_2 cell cycle delay, and that elevating cyclin B1 expression is part of the mechanism of action of pentoxifylline on G_2 blocked cells.

Numerous *in vitro* studies have shown that methylxanthines such as caffeine and pentoxifylline increase the toxicity of radiation and alkylating agents and give rise to dose enhancing factors in the range of 1.2–1.6 (Fingert *et al.* 1988, Vernimmen *et al.* 1994). Added at maximum expression of the radiation induced G_2/M cell cycle block, these drugs will shorten the cell cycle delay and rapidly restore normal cell cycle distribution (Kim *et al.* 1993) and inhibit repair (Link *et al.* 1996).

The entry of G_2-phase cells into mitosis is induced and regulated by the maturation- or mitosis-promoting factor (MPF), identified as a complex between cyclin B1 and $p34^{cdc2}$ kinase (reviewed by Norbury & Nurse 1992). The activity of the MPF complex is subject to strict regulation by dephosphorylation and phosphorylation of specific amino acid residues in the $p34^{cdc2}$ protein (reviewed by Murray 1992). In order to identify the molecular events which control the formation of the G_2 cell cycle delay, numerous attempts have been made to determine the expression of the cyclin B1 and $p34^{cdc2}$ proteins in response to DNA damage. Using synchronized cells, it was shown that irradiation suppresses cyclin B1 expression when cells go into the G_2 block (Muschel *et al.* 1991, Tsao *et al.* 1992, O'Connor *et al.* 1993, Ling *et al.* 1996). A study on the effects of caffeine on HeLa cells has shown that this drug elevates cyclin B1 expression while diminishing the cell cycle block (Bernhard *et al.* 1994a). Studies on asynchronous cells however, failed to demonstrate a significant reduction in cyclin B1 expression after irradiation (Villa *et al.* 1996, Cohen-Jonathan *et al.* 1997), whereas one study

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reported a dose-dependent increase in cyclin B1 expression concomitant with the rise in the G_2/M fraction in both synchronized and asynchronous cultures (Smeets *et al.* 1994).

In order to resolve these discrepancies, we wondered about the need of using synchronous cells and the complications arising from unscheduled expression of cyclins and other cellular proteins (Gong *et al.* 1995). The relationship between G_2 levels and cyclin B1 levels has not previously been measured. Using asynchronous HeLa cells, which produce a strong G_2 block and in which cyclin B1 expression is well characterized, we determined the cyclin B1/G_2 ratio over a timespan of 25 h after the induction of a G_2 cell cycle block and in response to the G_2 block abrogator pentoxifylline. The results demonstrate that there is indeed a large increase in cyclin B1 expression concomitant with the rise in G_2-phase fraction. However, when the cyclin B1 levels are expressed as the cyclin B1/G_2 ratios, irradiated cells show B1/G_2 ratios below control levels for the entire duration of the block. The cyclin B1/G_2 ratio recovers slowly as G_2 levels are restored to normal. When pentoxifylline is added at maximum expression of the G_2 block the G_2 levels rapidly decline, cells progress into mitosis showing a sharp rise of the cyclin B1/G_2 ratio to control levels. These results reflect on the molecular events which contribute to the formation of the radiation induced G_2 cell cycle delay and the mechanism of action of pentoxifylline as a G_2 delay abrogator.

MATERIALS AND METHODS

Asynchronous populations of HeLa cervical carcinoma cells were plated in McCoy's medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal calf serum (FCS) in 75 cm^2 cell culture flasks and cultured at 37°C in 95% O_2/5% CO_2. Samples in exponential phase were irradiated with 7 Gy of ^{60}Co γ-irradiation and allowed to grow for 10 h, which is the time needed for maximum G_2 block formation in HeLa cells. Pentoxifylline was then added to a final concentration of 2 mM. At different time intervals thereafter, ranging from 2–25 h, the pentoxifylline treated and control samples were trypsinized, fixed in 70% ethanol and stored at −20°C.

Determination of G_2 block

The determination of the time of maximum cell cycle block in G_2 was done by irradiating HeLa cells with 7 Gy of ^{60}Co γ-irradiation, and sampling cells at 2-hourly intervals for up to 25 h. The cells were fixed in 70% ethanol and stored overnight at −20°C. After washing in phosphate buffered saline (PBS), cells were resuspended in PBScontaining 10 μg of propidium iodide (Sigma, St. Louis, MI) and 0.1% RNase A. Samples were incubated for 20 min at 37°C prior to flow cytometric analysis.

Immunocytochemistry

Fixed cells were prepared for multiparameter flow cytometry to simultaneously measure total DNA content and cyclin B1 protein expression as previously described (Gong *et al.* 1994a). In short, cells were washed in PBS and treated with 0.25% Triton X-100 for 5 min on ice. After another wash in 5 ml PBS, the cell suspension (5×10^5 cells/100 μl) was incubated overnight at 4°C in a 1:400 dilution of mouse monoclonal anti-cyclin B1 antibody (Pharmingen clone GNS-1, San Diego, CA) in PBS/1% bovine serum albumin (BSA). The antibody was used to a final concentration of 0.25 μg/100 μl PBS/1% BSA. The next morning, cells were washed in PBS and incubated for 30 min at room temperature in a 1:40 dilution of fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Sigma) in PBS/1% BSA. The cells were washed again, resuspended in 10 μg/ml of propidium iodide

(PI) and 0.1% RNase A in PBS, and incubated for 20 min at room temperature prior to analysis. A negative control sample was prepared in a similar way, except that an isotype-specific antibody, mouse IgG1 (Sigma), was used instead of the cyclin B1 antibody.

Flow cytometry

To determine the time of maximum G_2 block and cyclin B1 levels, cells were stained with propidium iodide (PI) and aliquots were subjected to the anti-cyclin B1 antibody and FITC-conjugated second antibody. Samples were analysed on a Becton Dickenson (San Jose, CA) FACScan flow cytometer. Fluorescence data from 10000 events per sample were collected, stored and analysed using LYSIS II software. To determine the time of maximum G_2 block, samples were analysed for their red (PI) fluorescence which was displayed as a DNA histogram. Markers were placed in the G_2 area in order to determine the G_2 content at each postirradiation time-point.

Cyclin B1 expression and DNA data were displayed in dot plots of red (PI) vs. green (FITC) fluorescence representing total cellular DNA content and cyclin B1 expression, respectively. The doublet discrimination module was used to gate out cell doublets. The determination of G_2 fraction was done as described above, while the fraction of cells expressing cyclin B1 was determined by gating only cells which displayed a positive green (FITC) fluorescence. The threshold for cyclin B1 expression was defined using a gate window set on the negative control sample, which was prepared with the isotype-specific antibody IgG1, on all the treated samples. All experiments were repeated twice and essentially identical results were obtained.

Definition of cyclin B1/G2 ratios

These values were calculated by comparing the fraction of cells expressing cyclin B1 to the fraction of cells in the G_2 phase of the cell cycle for each time point measured after irradiation, and after the addition of pentoxifylline.

RESULTS

In HeLa cells (Fig. 1a), the radiation induced G_2 cell cycle block reaches a maximum at 10 h after a single dose of 7 Gy (Fig. 1b). From the dot-plot of red (PI) vs. green (FITC) fluorescence, it is clear that cyclin B1 is only synthesized in late S-phase and reaches maximum expression in G_2 (Fig. 2a). No cyclin B1 is seen in G_1-phase because of the rapid

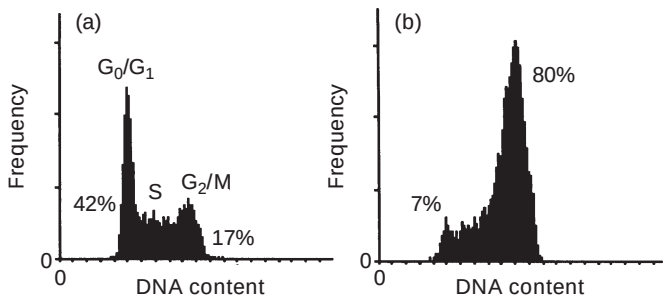

Figure 1. Flow cytometric histogram of the total DNA content of HeLa cells, indicating the normal cell cycle distribution (a) and the maximum formation of the G_2 cell cycle delay at 10 h after irradiation (b).

degradation of this protein in early mitosis. As the cells accumulate in G_2, there is a concomitant rise in cyclin B1 expression (Fig. 2b).

The increase in the G_2 fraction of the cell cycle during expression of the block and the decrease of this fraction as the block resolves over 35 h are shown in Fig. 3 (solid line). Addition of 2 mM pentoxifylline at 10 h after irradiation, when the G_2 delay had reached a

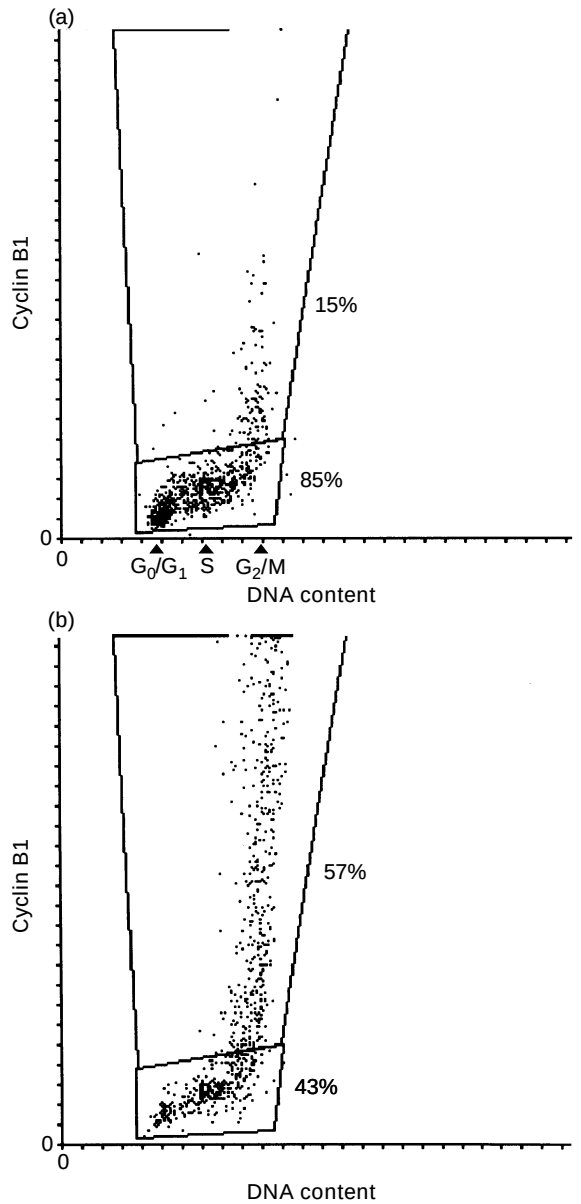

Figure 2. Dot plots of green fluorescence (FITC) on the vertical scale vs. red fluorescence (PI) on the horizontal scale, representing cyclin B1 and total DNA content, respectively, indicating the G_2-phase specific expression of cyclin B1 in HeLa cells before (a) and 10 h after irradiation (b).

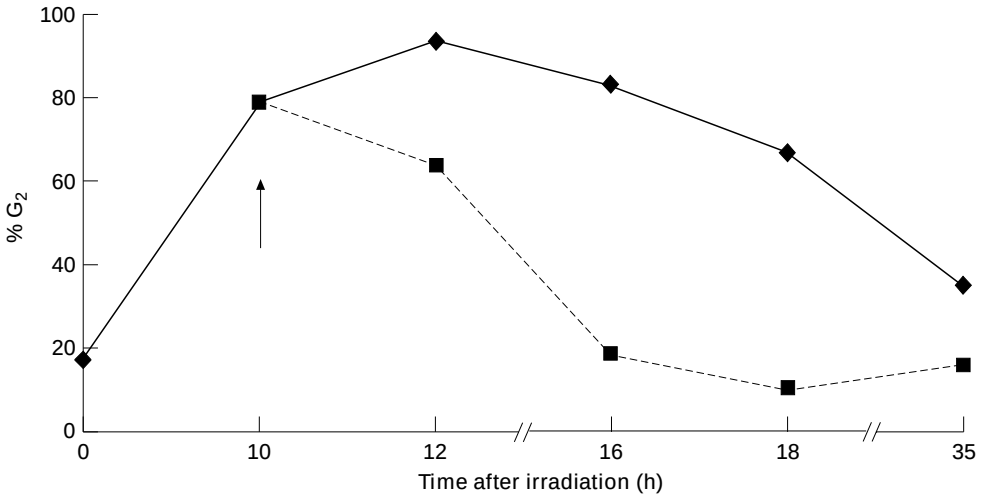

Figure 3. Increase of the G_2 fraction of the cell cycle during expression of the G_2 block and the decrease of this fraction as the block resolves over 35 h. The dashed line indicates the rapid abrogation of the block after the addition of 2 mM pentoxifylline at the 10-h time point (arrow).

maximum, rapidly abrogates the G_2 delay and cells return into cycle (Fig. 3, dashed line). Over the same time window, cyclin B1 content rises and parallels the increase of the G_2-phase fraction after irradiation (Fig. 4 — solid line), and the decrease after pentoxifylline treatment (Fig. 4 — dashed line).

When the ratio of cyclin B1 fraction vs. G_2 fraction is plotted, it is seen that cyclin B1 expression actually decreases at the induction of the G_2 block, and stays below control levels for the entire duration of the G_2 block (Fig. 5 — solid line). When pentoxifylline is added at

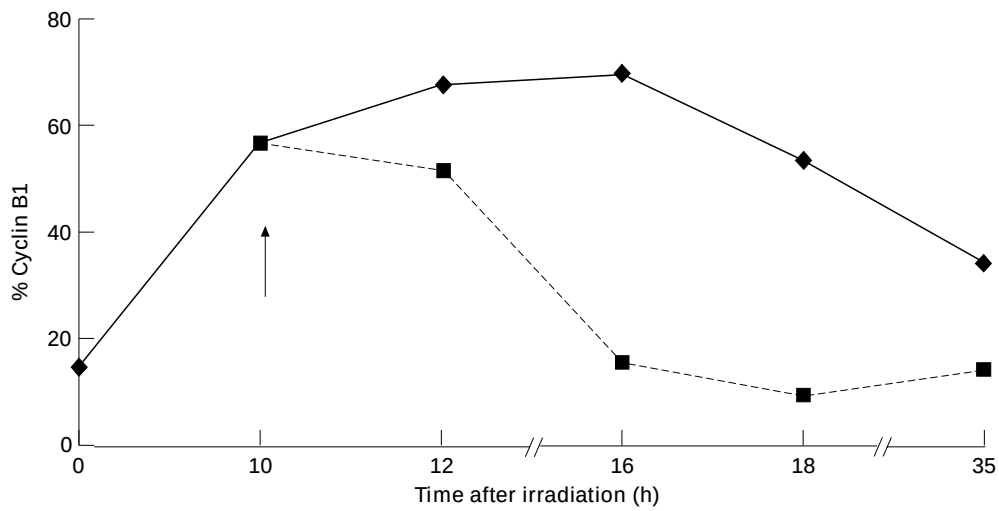

Figure 4. Increase of the cyclin B1 expression which parallels the rise of the G_2 fraction after irradiation. The abrogation of the G_2 block by pentoxifylline as indicated by the dashed line.

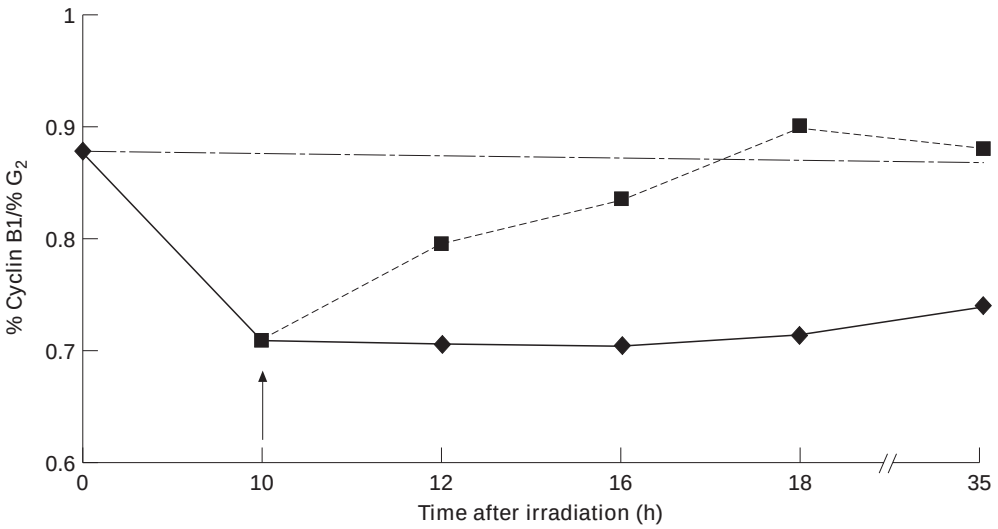

Figure 5. Cyclin B1/G_2 ratio plotted over 35 h after irradiation. This ratio is suppressed after irradiation at the formation of the G_2 block, and recovers slowly as the block resolves (———). After the addition of pentoxifylline (----), this ratio rapidly recovers to reach the control level (—·—·—) within 6–8 h.

10 h after irradiation, this ratio is rapidly restored to the level seen in the unirradiated control sample (Fig. 5 — top dashed line).

DISCUSSION

The simultaneous measurement of total DNA content and cyclin B1 expression allows for a quick and relatively easy way of directly correlating cyclin B1 expression to cell cycle phase. Most previous studies have employed synchronized cells and the more laborious Western blotting method to analyse for cyclin B1 expression (Muschel *et al.* 1991, Muschel *et al.* 1993, Bernhard *et al.* 1994a) and thus could not correlate cyclin expression and DNA content directly. Other authors have, however, indicated the value of flow cytometric analysis of asynchronous populations for this type of study (Sherwood *et al.* 1994). Our flow cytometric results revealed that cyclin B1 levels in HeLa cells begin rising in late S-phase and reach a maximum in G_2 phase. No cyclin B1 expression was seen in other cell cycle phases. This confirms that HeLa cells do not exert unscheduled cyclin expression often seen in other tumour cells (Gong *et al.* 1994b). The tendency of synchronized cells to show an unscheduled expression of cyclins and other cellular proteins has been demonstrated (Gong *et al.* 1995). For this reason we opted to use asynchronously growing undisturbed cell cultures.

Our results demonstrate that the down-regulation of cyclin B1 expression plays a role in the formation of the radiation-induced cell cycle delay in the G_2-phase. This suppression of cyclin B1 expression probably prevents the optimum formation of the mitosis- or maturation-promoting factor (MPF) which consists of a complex between the cyclin B1 protein and the $p34^{cdc2}$ kinase protein, and is essential for cells to cross the threshold from G_2 into mitosis. It has been hypothesized that the accumulation of cells in G_2 phase may have a protective function, allowing time for DNA repair before the cell re-enters mitosis (Tobey 1975). Our conclusions are in agreement with that of other studies on synchronized cells which have

relied on different methods of analysing cells for cyclin expression after exposure to irradiation (Muschel *et al.* 1991, Bernhard *et al.* 1994a) and after treatment with DNA damaging agents like camptothecin, etoposide and nitrogen mustard (Maity *et al.* 1996). A dose-dependency of cyclin B1 suppression observed by Villa *et al.* (1996) cannot be refuted since we used a constant dose of 7 Gy throughout. Our finding of an increase in cyclin B1 expression concomitant with the increase in G_2 fraction is in agreement with data from Smeets *et al.* (1994). However, the percentage of cells in G_2-phase in asynchronous cells is small (10–15%) and virtually all the cyclin B1 originates from this G_2 fraction. When these cells are irradiated, the G_2 levels rise to $\approx$80% and this explains the massive rise in cyclin B1 (Fig. 2). However, when the cyclin B1 content is expressed in relation to the G_2 content, it is seen that irradiation in fact reduces the B1/G_2 ratio to below control levels. When we followed this ratio at regular intervals over 25 h, we found that it slowly returns to normal, concomitant with the recovery of the cell cycle. The approach used here solves two problems: it eliminates the need for synchronized cells, and it puts the cyclin B1 levels in perspective to the G_2 fraction.

The addition of the methylxanthine drug pentoxifylline to irradiated cells blocked in the G_2-phase has been shown to cause a rapid overrride of the block and return of the cells to a normal cell cycle distribution (Musk & Steel 1990). These cells presumably have less time for DNA repair and are forced to undergo mitosis before completion of DNA repair. This may lead to the increased cell kill observed and is in agreement with results on the use of pentoxifylline as a radiation adjuvant (Vernimmen *et al.* 1994). We now show that abrogation of the G_2 block by pentoxifylline restores the cyclin B1/G_2 ratio to control levels within 6–8 h after addition of the drug. Our results are in agreement with other studies on the influence of pentoxifylline (O'Connor *et al.* 1993) and caffeine (Bernhard *et al.* 1994b) on cyclin B1 expression in nitrogen mustard treated and irradiated cells, respectively. The induction of cyclin B1 expression by pentoxifylline in G_2 blocked cells resembles data on a dexamethasone-inducible promotor which abrogates the G_2 block (Kao *et al.* 1997). We therefore conclude that the level of cyclin B1 is indeed a controlling factor in the length of the G_2 delay.

Formation of the MPF by the availibility of sufficient quantities of cyclin B1 and $p34^{cdc2}$ is only part of the mechanism by which cells recognize DNA damage and control the cell cycle at the G_2 checkpoint. This complex has to be activated by different kinases and phosphatases in order to be functional (reviewed by Murray 1992). The decrease in cyclin B1 expression below control levels after induction of the G_2 block is rather small and hence may not be solely responsible for the formation of the G_2 delay. The move of cyclin B1 to the cell nucleus may also be an important event (Kakino *et al.* 1996) and has been shown to drop sharply after irradiation (Smeets *et al.* 1994). Various studies on the expression of the $p34^{cdc2}$ protein (Lock & Ross 1990, Lock 1992), the activity of the MPF as regulated by $p34^{cdc2}$ phosphorylation (Ling *et al.* 1996, Orlandi *et al.* 1996) and the localization of cyclin B1 (David-Pfeuty & Nouvain-Dooghe 1996, Li *et al.* 1997) and $p34^{cdc2}$ (Cohen-Jonathan *et al.* 1997) have indicated other levels of control at the G_2 checkpoint. Answers to the role of cdc25 phosphatase and mik1/wee1 kinase activity which plays a crucial role in the activation of the MPF may help to unravel the complexities of the G_2 delay mechanism.

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