Influence of irradiation and pentoxifylline on histone H3 phosphorylation in human tumour cell lines

A. Binder and L. Bohm

Department of Radiation Oncology, University of Stellenbosch, Faculty of Health Sciences and Tygerberg Hospital, Tygerberg, South Africa

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Abstract. Phosphorylation of histone H3 at Ser-10 correlates with chromatin condensation and this amino terminal modification is now recognized as a specific marker of mitosis. We have monitored the appearance of cells showing histone H3 phosphorylation in four human tumour cell lines to identify cell cycle progression after irradiation. In the human melanoma cell lines Be11 and MeWo and in the squamous cell carcinoma lines 4197 and 4451 a dose of 7 Gy of Co- γ irradiation increases the number of cells binding anti-histone H3-P antibody 1–8-fold in a p53-independent manner. In the p53 mutant cell lines MeWo and 4451 H3-P phosphorylated cells can be detected as early as 30 min and show a maximum 1 h post-irradiation. In the cell lines Be11, 4197 and 4451 the early wave of H3 phosphorylated cells is followed by a second wave, which reaches a maximum 4.5–7 h post-irradiation and then declines. These events are attributed to damage-induced cell cycle blocks in the G₁ and G₂ phase of the cell cycle. Addition of the dose modifying drug pentoxifylline before irradiation increases the appearance of cells showing early and the late H3 phosphorylation. When pentoxifylline is added 12–24 h post-irradiation when the cell cycle blocks have reached their maximum the appearance of cells with phosphorylated H3 increases 3–5-fold in the p53 mutant cell lines MeWo and 4451. These observations are consistent with the function of the drug as a G₂ block abrogator. The large H3 phosphorylation signal in p53 mutant cells is consistent with early entry of a cohort of G₂ cells into mitosis. The smaller H3-P signal in p53 wild type cells correlates with the lower proportion of stable G₂ populations in G₁ blocked cells. These results indicate that pentoxifylline influences the appearance of histone H3 phosphorylated cells in a manner strongly dependent on the number of cells in G₂ phase. This suggests that addition of pentoxifylline indeed abrogates the G₂ block and thereby facilitates early entry into mitosis.

INTRODUCTION

The amino-terminal tails of histone H3 and H4 are not confined to the oligomeric histone core but extend beyond the range of the particle where they influence the interactions between

nucleosomes (Luger *et al.* 1997a,b). Their exposed location makes these tail domains accessible to various modifications on lysine residues (Bohm & Crane-Robinson 1984; Libertini *et al.* 1988). N-terminal histone acetylation has been shown to destabilize the 30 nm solenoid (Allan *et al.* 1982; Davie & Chadee 1998) and is associated with transcriptional activation (Wolffe 1992; Vettese-Dadey *et al.* 1996). During a normal cell cycle, but restricted to the G₂/M transition histone H3 phosphorylation at Ser-10 correlates with chromatin condensation and this modification has been widely used as a specific marker of mitosis (Norbury & Nurse 1992; Wei *et al.* 1999). The G₂/M transition is facilitated by activation of the mitosis promoting factor consisting of the p34^{cdc2}kinase-cyclin B1 complex which initiates chromosome condensation (Shimada *et al.* 1998). This event closely correlates with histone H3 phosphorylation (Juan *et al.* 1999). Mitosis-specific H3 phosphorylation occurs during M phase (Gurley *et al.* 1978; Sauve *et al.* 1999) but is also observed in premature chromosome condensation at Ser-10 (Ajiro *et al.* 1983; Ajiro & Nishimoto 1985). There is now little doubt that phosphorylation of histone H3 is associated with chromatin condensation, which is observed, when cells transverse from G₂ into M-phase (Ajiro *et al.* 1983; Ajiro & Nishimoto 1985; Sauve *et al.* 1999). Expression of cyclins A and B1 proteins essential for cell cycle progression is also associated with H3 phosphorylation suggesting a role of this modification in transcription or translation (Juan *et al.* 1999). The mitosis-associated phosphorylation of H3 is initiated in pericentromeric heterochromatin in late G₂ interphase cells and it has been recognized that there is a precise spatial and temporal correlation between H3 phosphorylation and initial stages of chromatin condensation (Hendzel *et al.* 1997; Goto *et al.* 1999).

The methylxanthine drug pentoxifylline is a potent phosphodiesterase inhibitor (Zabel *et al.* 1993; Reuter & Wallace 1999). Both pentoxifylline and the closely related drug caffeine influence cell cycle check points by activating the mitosis promoting factor MPF (O'Connor *et al.* 1993; Stewart *et al.* 1997; Theron & Bohm 2000) and abrogating G₂ blocks induced by irradiation (Crompton *et al.* 1993; Theron & Bohm 1999, 2000; Theron *et al.* 2000) or genotoxins (Fingert *et al.* 1986; O'Connor *et al.* 1993; O'Loughlin *et al.* 2000). Caffeine also induces mitosis in S-phase-arrested hamster cell lines (Franchitto *et al.* 1998) and apoptosis in S-phase arrested human cells (Downes *et al.* 2000). It therefore appears that more than one signal transduction pathway is affected by the drug (Yoshida *et al.* 1997). Chromatin condensation seen in apoptosis and in mitosis differ in that apoptosis is associated with the aggregation of heterochromatin (Hendzel *et al.* 1998; Ajiro 2000) while mitosis shows de novo chromatin condensation of euchromatin which is not seen in apoptosis (Hendzel *et al.* 1998).

In the present study we have examined the effect of pentoxifylline on histone H3 phosphorylation in a range of human tumour cell lines of defined p53 status. We report the post-irradiation period is characterized by the appearance of H3 phosphorylated cells in preparation for mitosis, whose number increases when the cells are released from the cycle blocks. We demonstrate that the effects of abrogation of the G₂ block by pentoxifylline can be tracked by histone H3 phosphorylation to assess mitotic progression and re-entry of cells into the cell cycle.

MATERIALS AND METHODS

Cell culture

The human melanoma cell lines Be11 (p53 wild type), MeWo (p53 mutant), and the human squamous carcinoma cell lines (SCC) 4197 (p53 wild type) and 4451 (p53 mutant) were kindly

provided by F. Zoelzer and C. Streffer, University of Essen, Germany. Cells were grown in minimal essential medium (MEM) with 20% fetal bovine serum (FBS), 2 ml/L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/mL) in 75 cm² culture flasks incubated at 37 °C in 95% O₂–5% CO₂.

Cell irradiation and treatment with pentoxifylline

Exponential growing cells in 25 cm² cell culture flasks were irradiated with a dose of 7 Gy ⁶⁰Co-γ-irradiation using a Theratron at a dose rate of 1 Gy/min. Cells were treated with 2 mM pentoxifylline (PTX, Hoechst, Germany), 5 ml/100 mg directly after irradiation or alternatively at the time of G₂ block maximum which differed for each cell line, and harvested at indicated time intervals. Samples were prepared for flow cytometric analysis by trypsinization, centrifugation and fixation as described below.

Immunochemical detection of BrdU

Staining of cells for DNA content and BrdU incorporation was as described by Wilson (1994). Irradiated cells were pulse-labelled for 15 min in 10 µM BrdU. The BrdU-labelled cells were fixed in 70% ethanol (at –20 °C). Denaturing the cells in 2 M HCl/0.2 mg/ml pepsin was followed by staining the cells with a mouse anti-BrdU antibody and an fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG second antibody. After resuspension in a PI (Sigma, St Louis, MO) and RNase (Sigma) staining solution, flow cytometric analysis was done on a FACScan (Becton Dickinson, San Diego, CA) flow cytometer at 488 nm. Red fluorescence (PI) was collected as a linear signal and recorded as a measure of the total DNA content. Cell doublets were gated out by processing red fluorescence into area and width. Green fluorescence (anti-BrdU-FITC) was recorded on a log scale. The BrdU-labelled S-phase cells were clearly identified by their green fluorescence and were lying between the G₁ and the G₂ populations. The latter two populations could also be separately identified by their lack of BrdU uptake and the difference in their DNA content. The BrdU positive part of the dot plot was used to calculate S phase cells using ModFit and CellQuest software.

Immunochemical detection of p21^{WAF1}

To confirm the p53 wild type status of the given cell lines, the p53 dependent p21^{WAF1} induction was used. Cells were irradiated and harvested at 2-h intervals for up to 24 h. Detection of p21^{WAF1} was completed according to a modified (Binder *et al.* 2001) protocol of Deptala *et al.* (1999). Cells were fixed in 1% methanol-free formaldehyde at 4 °C for 15 min, permeabilized with 80% ethanol at –20 °C. After fixation and reswelling in PBS at room temperature cells were lysed in 0.25% Triton-X-100 on ice for 5 min. Cells were washed with 1% BSA/PBS followed by an over night incubation at 4 °C with the anti-p21^{WAF1} antibody or a negative isotype control mouse anti-IgG1. Cells were then stained with an FITC-conjugated goat anti-mouse IgG second antibody (Sigma). After resuspension in a PI and RNase staining solution, flow cytometric analysis for the DNA content and the p21^{WAF1} induction was carried out as described above. For the p21^{WAF1} induction Cell Quest software was used.

Immunochemical detection of histone H3 phosphorylation

Phosphorylation of histone H3 on Ser-10 (H3-P) correlates with chromatin condensation at mitosis (Juan *et al.* 1999). The anti-H3-P monoclonal antibody (ONCOR, Gaithersburg, MD) was kindly provided by Dr Z. Darzynkiewicz (New York Medical College, Valhalla, NY). To assess the influence of pentoxifylline on H3 phosphorylation, cells were irradiated, treated with 2 mM pentoxifylline directly after irradiation or alternatively at the time of G₂ block maximum

which differed for each cell line, and harvested at indicated time intervals for up to 48 h. Samples were prepared for flow cytometric analysis as described by Juan *et al.* (1999). In short, cells were fixed in 1% methanol-free formaldehyde for 15 min, washed and fixed in 80% ethanol overnight. Lysing in 0.25% Triton-X-100 on ice for 5 min was followed by staining with the anti-H3-P mouse monoclonal antibody mouse anti-IgG1 or a negative isotype control (Sigma) and a FITC-conjugated goat anti-mouse IgG second antibody (Sigma). After resuspension in a PI and RNase staining solution, flow cytometric analysis was carried out as described above. Green fluorescence (anti-H3-P-FITC) and red fluorescence (PI) were collected on a linear scale. For controls, cells were treated with alkaline phosphatase (Sigma) at 4 units/100 µl or blocked in mitosis by nocodazole (0.4 µg/ml) treatment for 24 h. The appearance in frequency of cells with phosphorylated H3-P was measured by using Cell Quest software.

Data evaluation

All experiments were repeated at least three times, and the data are given as a mean $\pm$ standard deviation (SD) for the independent experiments.

RESULTS

In the MeWo and 4451 cell lines the p53 mutations are identical and consist of a G-to-A transition in codon 258 of exon 7. This leads to the substitution of glutamine with lysine (Zoelzer *et al.* 1995). We have previously demonstrated that the p53 status in the two p53 wild type cell lines 4197 and Be11 is indeed intact as shown by expression of the p53 product p21^{WAF}. The p21^{WAF1}-labelled cells were identified by the presence of a green fluorescence, which was selective to the G1 population. The S and the G2 populations were clearly non reactive with the p21^{WAF1} antibody (Binder *et al.* 2000). The H3-P-positive cells were identified by their green fluorescence and were characterized by a G₂/M DNA content (Fig. 1c). Negative controls were cells treated with alkaline phosphatase. Cells blocked in mitosis by nocodazole for 24 h served as positive controls. When irradiated with 7 Gy of ⁶⁰Co-γ irradiation, the p53 mutant MeWo and 4451 cell lines showed binding of anti-H3-P antibody, which peaked 30–60 min post-irradiation. In 4451 cells but not in MeWo cells this was followed by a second larger peak 4.5–7 h post-irradiation. The second peak was also seen in the Be11 and 4197 cell lines (Fig. 1a). Addition of pentoxifylline before irradiation was of little to no consequence to 4451 cells in the first peak

Figure 1. (a) Influence of pentoxifylline on the frequency of cells binding the anti-H3-P monoclonal antibody in Be11, MeWo, 4197, and 4451 cells. Irradiation increases anti-H3-P antibody binding in a p53 independent manner and shows an early maximum at 30–60 min and a late maximum at 4.5–6.5 h post-irradiation. Addition of pentoxifylline before irradiation (arrow) increases the appearance of cells showing early and late H3-phosphorylation. This effect tends to be larger in p53 mutant cells. ♦, irradiation only; ■ irradiation and pentoxifylline. (b) Influence of pentoxifylline on binding of the anti-H3-P monoclonal antibody in Be11, MeWo, 4197, and 4451 cells. A G₂-block was induced by 7 Gy of irradiation. Arrow indicates addition of pentoxifylline. Abrogation of the G₂ block by pentoxifylline increased the frequency of H3-P positive cells within 2 h of drug addition 1.7–2.2-fold for the wild type p53 cell lines, 3–5-fold for the p53 mutant lines. The H3-P-positive cells were identified by flow cytometry as shown in (c). ♦, irradiation only; ■ irradiation and pentoxifylline. (c) Multivariate analysis of 4197 cells stained for DNA content, anti-H3-P reactivity for either control cells incubated with isotypic IgG instead of anti-H3-P (left), control cells incubated with anti-H3-P (centre) and cells 14 h post-irradiation exposed to 2 h of pentoxifylline (right), detected immunocytochemically. Cells (4197) were fixed and stained for DNA content with PI (red fluorescence emission), for H3-P with secondary antibody conjugated with FITC (green fluorescence), as described in Materials and Methods. The cells reactive with anti-H3-P-antibody were selected (gated).

Figure 2. Irradiation-induced changes in the percentage of S-phase cells estimated from DNA content frequency histograms. Irradiation induces a G_1 block and a decline of S-phase cells in 4197 (a) and Be11 (b); both are p53 wild type cell lines. In the two p53 mutant lines 4451 (a) and MeWo (b) the frequency of S-phase cells increases after irradiation showing a maximum within 0.5 h. ●, p53 mutant cells; ■, p53 wild type cells.

but induced a 7.5-fold increase (0.6–4.5) in percentage of H3-phosphorylated cells in the second peak. In the melanoma cells the second increase between 4.5 and 7 h was particularly pronounced in the p53 mutant MeWo cell line. Thus, MeWo p53 mutant cells underwent a 4.3-fold increase (2.1–5.2) in frequency of H3 phosphorylated cells in the first hour and a 5.2-fold increase (0.92–4.3) at 7 h post-irradiation. This compares with only a 2-fold increase (4.1–8.2) in the second peak of Be11. When the cell lines were irradiated with 7 Gy and pentoxifylline was added later at the G_2/M maximum only one peak was observed rising 1.7–2.2-fold (0.6–1.0) in Be11 and (7.9–17.7) in 4197 p53 wild type cells and 2.8–5-fold (1.3–6.7) in MeWo and (3.8–10.6) in 4451 p53 mutant cells (Fig. 1b). Estimation of the percentage S-phase cells from DNA histograms showed a marked decline of S-phase cells in the p53 wild type cells as expected for cells blocked in G_1 (Fig. 2). In the p53 mutant cells on the other hand the frequency of S-phase cells was found to increase after irradiation showing a maximum at 1 h post-irradiation. When S-phases were identified in 4451 p53 mutant cells by BrdU labelling it was observed that their frequency declined from 50 to 28% at 24 h post-irradiation at the time when the percentage of G_2 cells had reached a maximum (Fig. 3). At 31 h post-irradiation when the cell cycle block was resolved and G_2 cells re-entered G_1 phase, the percentage of cells incorporating BrdU was at its lowest (19%) and then increased to 31% (Fig. 3). Addition of pentoxifylline 24 h post-irradiation at the G_2 maximum and incubation of cells in the presence of the drug for 7 h induced a decrease in the G_2 population from 64 to 24% and a nearly complete restoration of the G_1 population. The frequency of cells in G_1 overshoot from 39% in controls to 59% seen in the presence of pentoxifylline for 7 h declined to control values when pentoxifylline was present for 8 h. After 8 h pentoxifylline treatment the S-phase content had also returned to normal levels of approximately 50% (Fig. 3).

Figure 3. Flow cytometric analysis of DNA content in irradiated 4451 cells. Lower panels show S-phase progression. Upper panels show induction of G₂ block at 24 h, recovery at 31 and 32 h and influence of pentoxifylline added at 24 h and left for 7 or 8 h as compared to 32 h control. The lower panel shows dot plots of DNA content (x-axis/red fluorescence) versus BrdU content (y-axis/green fluorescence). Suppression of DNA synthesis is shown at 24 h, recovery begins at 31 h and accelerated recovery induced by 7 h presence of pentoxifylline is evident.

DISCUSSION

Addition of pentoxifylline prior to irradiation induces a marked increase of radiotoxicity. Dose modifying factors in the range of 1.2–1.9 have been observed especially in p53 mutant cell lines (Kim *et al.* 1993; Vernimmen *et al.* 1994; Li *et al.* 1998; Theron *et al.* 2000). Addition of pentoxifylline 12–24 h post-irradiation at the time of maximal G₂ block served the purpose of abrogating this block (Theron & Bohm 1998, 2000; Theron *et al.* 2000). Under these conditions the dose modifying factor was extremely low and in the range of 1.0–1.1 (Theron *et al.* 2000; Binder *et al.* 2000). Nevertheless numerous authors have suggested that early entry into mitosis before the normal recovery of the cell cycle block will reduce clonogenic survival and hence explain the dose modifying effect of pentoxifylline observed when drug and irradiation are applied together (Lock *et al.* 1994; Bernhard *et al.* 1996; Jackson *et al.* 2000). It was therefore of great

interest to examine the H3 phosphorylation signal in these two situations. The basic observation is that irradiation changed the number of H3-phosphorylated cells in both cell types and that these events were further modulated by pentoxifylline (Fig. 1a). In p53 wild type cells 4197 and Be11 which entered a G₁ block 12 and 16 h post-irradiation, respectively, entry into the block was preceded at 5–6.5 h by an increase of the number of H3 phosphorylated cells (Fig. 1a). In p53 mutant 4451 cells the 4.5–7 h peak of H3-P positive cells was lower and was preceded by a H3-P peak 30–60 min post-irradiation. In the p53 mutant MeWo cell line only the first peak but no second peak was observed. The appearance of a large 5–6.5 h H3-P peak in both p53 wild type cells may be due to their faster cell cycle and to the presence of cells still progressing from G₂ to M before being blocked in G₁. The less expressed peak of H3-P positive cells, at 4.5–7 h or its absence in the p53 mutant cell line may be due to the slower accumulation of cells in G₂ shown by the fact that fewer cells were progressing from G₂ to M (Fig. 1a). Our experiments demonstrate that administration of pentoxifylline prior to irradiation increased the frequency of histone H3 phosphorylated cells occurring 4.5–7 h postirradiation 5–8-fold in p53 mutant cell lines (Fig. 1a). When the drug was added post-irradiation, at the time of maximal block in G₂, only one peak was apparent within 1–2 h of pentoxifylline addition. This peak represented a 3–5-fold increase in p53 mutant cells and a 2-fold increase of H3-phosphorylated cells in p53 wild type cell lines (Fig. 1b). The high number of cells with phosphorylated H3 in the p53 mutant cell line correlated with the larger number of cells in G₂ phase compared with the p53 wild cell lines. Likewise, the less pronounced peak of H3-positive cells in p53 wild cell lines is consistent with a lower proportion of G₂ cells. Addition of pentoxifylline at the time of maximal block in G₂ to p53 mutant cell lines reduced the G₂ content, and BrdU incorporation demonstrated re-entry into S-phase after 7 h (Fig. 3). These observations are consistent with the role of pentoxifylline as a G₂ block abrogator and indicates that the status of p53 (wild versus mutant) plays a minor role in the abrogation. The decline in frequency of H3 phosphorylated cells under these conditions after 7 h of incubation with pentoxifylline (Fig. 1b) is consistent with a role of H3 phosphorylation in mitotic progression (Chadee *et al.* 1999; Cheung *et al.* 2000). The early peak of cells with phosphorylated histone H3 seen already 30–60 min postirradiation in p53 mutant cells is induced by pentoxifylline only in the melanoma cell line (Fig. 1a). It is possible that its induction in the SCC cell line did in fact occur, but was missed by several minutes and required more frequent sampling to be detected. It is possible that the early cohort of H3 phosphorylated cells was a consequence of activation and recruitment of cyclins and cyclin dependent kinases required when the cell cycle blocks were resolved. An involvement of histones in re-entry into the cell cycle is documented for histone H1 (Samake & Smith 1997). The induction of cyclin B is paralleled by an increase in cyclin B1-specific associated histone H1 kinase activity which reverses a radiation-induced G₂ delay (Ianzini & Mackey 1997; Kao *et al.* 1999). The accumulation of cyclin B1 in late G₂ phase of the cell cycle and nuclear translocation is a prerequisite of initiation of mitosis (Theron & Bohm 1998; Toyoshima *et al.* 1998; Porter *et al.* 2000; Theron *et al.* 2000). Recent evidence indicates that caffeine abolishes the G₂/M checkpoint by inhibiting ataxia telangiectasia mutant (ATM) kinase activity (Zhou *et al.* 2000). Both caffeine and pentoxifylline inhibit the ATM kinase activity, the enzyme required to phosphorylate Chk2/Cds1 at threonine 68, thereby reducing the radiation-induced ATM-dependent Chk2/Cds1 activation and phosphorylation (Sarkaria *et al.* 1999; Zhou *et al.* 2000). Radiotoxicity is markedly enhanced only in p53 mutant cell lines and only when pentoxifylline is present at the time of irradiation shown by dose modifying factors in the region of 2–14.5 at 8 Gy (Theron *et al.* 2000). Addition of the G₂ block abrogator pentoxifylline at the G₂ maximum promotes mitosis (Theron & Bohm 1998, 2000; Binder *et al.* 2000) but does not enhance radiotoxicity (Theron *et al.* 2000). We demonstrate here that addition of pentoxifylline to G₂ blocked cells is associated with a large

H3-P signal, which far exceeds the signal in the G₁ blocked p53 wild type cell lines (Fig. 1b). It therefore appears that mitotic progression cannot be the explanation for the mechanism of action of pentoxifylline as a dose modifier. That promotion of mitosis 20 h postirradiation is of no consequence for cell survival is consistent with repair kinetics showing that 95% of repair has been accomplished 2 h postirradiation (Dikomey *et al.* 1998; Roos *et al.* 2000). The fact that presence of the drug at the time of irradiation enhances radiotoxicity therefore would allow for a role of pentoxifylline in repair. We have demonstrated by constant field gel electrophoresis (CFGE) that repair of DNA damage induced at high dose is indeed suppressed by pentoxifylline irrespective of p53 status (Theron *et al.* 2000).

The foregoing results on the appearance of H3 phosphorylated cells after irradiation demonstrate that G₂ block abrogation by pentoxifylline is indeed associated with mitotic progression. The data also add further weight to our conclusion (Theron *et al.* 2000) and other results on caffeine (Ribeiro *et al.* 1999; Valenzuela *et al.* 2000) that mitotic progression cannot be the explanation for the enhancement of radiotoxicity by pentoxifylline.

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