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Variation in sensitizing effect of caffeine in human tumour cell lines after γ-irradiation

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Abstract

Background and purpose: We have investigated whether the protective role of the G2 checkpoint has increasing importance when the p53-dependent G1 checkpoint is inactivated.

Materials and methods: We have studied the differential effect of caffeine by clonogenic assays and flow cytometry in three human tumour cell lines with different functionality of p53 protein.

Results: The radiosensitizing effect of caffeine (2 mM) expressed itself as a significant decrease in surviving fraction at 2 Gy and a significant increase in α-values in RT112 and TE671, both with non-functional p53. However, no radiosensitizing effect was seen in cells with a normal p53 function (MCF-7 BUS). Two millimoles of caffeine also caused important changes in the cell cycle progression after irradiation. MCF-7 BUS showed a G1 arrest after irradiation and an early G2 arrest but those cells that reached the second G2 did not arrest significantly. In contrast, TE671 exhibited radiosensitization by caffeine, no G1 arrest, a G2 arrest in those cells irradiated in G2, no significant accumulation in the second G2 but an overall delay in release from the first cell cycle, which could be abrogated by caffeine. RT112 was similar to TE671 except that the emphasis in a G2 arrest was shifted from the block in cells irradiated in G2 to those irradiated at other cell cycle phases.

Conclusion: The data presented confirm that p53 status can be a significant determinant of the efficacy of caffeine as radiosensitizer in these tumour cell lines, and document the importance of the G2 checkpoint in this effect.

Keywords: Radiation; Caffeine; Cell-cycle; p53

1. Introduction

The importance of cell cycle control in the response of cells to DNA-damaging agents is widely accepted. Mammalian cells show a complex cellular response to DNA damage including activation of genes involved in cell cycle arrest, DNA repair and apoptosis. Cell cycle arrest following DNA damage is mediated by a series of negative feedback checkpoint systems that operate in late G1 and G2 phases and during the S phase. Cell cycle checkpoints in G1 and G2 phases protect DNA-damaged cells by delaying entry into the critical phases of the cell cycle: S and M phases, respectively [17,10].

X-irradiation tends to cause an arrest of the cell cycle at both the G1/S and G2/M boundaries. Many early studies demonstrated that the exposure of mammalian cells to ionizing irradiation prolongs both S and G2 phases [28,19]. A G1 delay following ionizing irradiation was first observed by Little [20]. In some cell types, signals arising from damaged DNA lead to activation of the p53 response pathway through an increased half-life of p53 resulting in cell cycle arrest, DNA repair or apoptosis [9,12]. Studies by McIlwrath et al. [23] and Siles et al. [34] showed that p53 function and G1 arrest after irradiation correlated with radiosensitivity in a series of human tumour cell lines, although this has not been a universal finding [31]. However, the role of p53 in G1 arrest was reinforced when the transfection of wild-type p53 into various tumour cell lines induced G1 accumulation under different conditions [1,21]. Some studies suggest that p53 could be also involved in the G2/M restriction point but

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G2/M arrest following X-irradiation has been observed in p53-deficient cells [36,30]. In addition G2 arrest could be sustained only when p53 is present in the cell and capable of transcriptionally activation of the cyclin-dependent kinase inhibitor p21. Both proteins p53 and p21 appear to be essential for maintaining the G2 checkpoint in human cells [3].

It has been suggested that the magnitude of the G2 delay after the treatment might be a critical determinant of cellular radiosensitivity [37]. Part of the evidence for this comes from the observation that abrogation of the G2 delay with methylxanthines such as caffeine (1,3,7-trimethylxanthine) and pentoxifylline results in increased cellular radiation and chemosensitivity [2,27,38]. However, caffeine-mediated radiosensitizion is not always associated with a change in cell cycle progression [26] and it has been suggested that caffeine may affect repair directly [26] and/or prevents the inhibition of DNA synthesis by radiation [39,18].

Nevertheless, the primary effect of caffeine is believed to be due to its ability to overcome the radiation-induced block at the G2/M phase of the cell cycle [4,32]. Recently, it has been shown that the sensitization of X-ray-induced cell killing by caffeine is greater in cells lacking the function of p53, compared with p53 wild-type cells [30,13,8]. It is suggested that a functional p53 system places emphasis on radiation-induced blocks at the G1/S checkpoint so that the G2/M checkpoint becomes less important. However, when the p53 response is inactive then the G2/M checkpoint is more important so that caffeine can exert a greater effect. This clearly has potential importance if tumour cells with a reduced p53-mediated G1 arrest are sensitized by caffeine to a greater extent than the surrounding normal tissues. Such a change in therapeutic ratio has obvious desirability in clinical radiotherapy.

In this study we have investigated whether the protective role of the G2 checkpoint has increasing importance when the p53-dependent G1 checkpoint is inactivated, by examining the differential effect of caffeine in three human tumour cell lines with different functionality of p53 protein and different clonogenic survival after irradiation.

2. Materials and methods

2.1. Cell culture

Three human tumour cell lines were used in this study. A human breast cancer cell line MCF-7 [35], named herein MCF-7 BUS, was grown in 10% foetal bovine serum-supplemented Dulbecco's modified Eagle's medium (FBS–DMEM, Sigma, St. Louis, MO) with penicillin (100 units/ml) and streptomycin (0.1 mg/ml). RT112 was derived from a human bladder carcinoma [22] and TE671 [6] from a human rhabdomyosarcoma. The latter two cell lines were grown in Ham's F12 medium supplemented with 10% foetal bovine serum, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). All cells were incubated at 37°C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Freedom from mycoplasma contamination was checked regularly by testing with Hoechst 33528 dye (Sigma, St. Louis, MO).

The functionality of p53 protein in these three cell lines was assessed after irradiation using flow cytometry. The p53-dependent G1-phase cell cycle checkpoint following ionizing radiation is one of the most significant p53 stress-related functions. The pattern of cell cycle progression and the levels p53 protein after irradiation corresponding to MCF-7 BUS and RT112 were published previously [34]. For TE671 time-course experiments of cell cycle distribution were done after 2 and 8 Gy. TE671 cells were arrested only in G2 but not in G1. According to these criteria MCF-7 BUS showed a functional p53 protein and RT1 12 and TE671 non-functional p53 protein.

2.2. Irradiation and colony formation

Cell survival following ionizing radiation was measured by clonogenic assay in monolayer. Cells were harvested and suspended in full culture medium. Single-cell suspensions were plated out at appropriate densities in triplicate. In all the experiments, cells in exponential growth were irradiated using a ^{60}Co source at a dose rate of 1.60 Gy/min. Irradiations were performed 4 h after plating when cells were attached. Caffeine (Sigma, St. Louis, MO) was dissolved in culture medium at a final concentration of 2 mM. It was added 30 min prior to irradiation and left on the cells for 24 h. After the caffeine was removed, cells were incubated in complete culture medium for 15–20 days after irradiation. Colonies of at least 50 cells were scored as surviving cells. Survival data were fitted using the linear-quadratic model [$\ln SF = -(\alpha D + \beta D^2)$], which has two components of cell killing: one is proportional to dose (αD) and the other is proportional to the square of the dose (βD^2).The α and β-parameters were determined by non-linear regression analysis. Three separate experiments were done for each cell line.

2.3. Cell cycle analysis

Cells were processed in the same way as colony assays. Cells were in exponential growth at the time of the irradiation and caffeine was also dissolved in culture medium at a concentration of 2 mM. It was added 30 min prior to irradiation and maintained for 24 h.

For each cell line $1–2 \times 10^5$ cells were plated into 25-cm^2 tissue culture flasks. After a period of 24 h, cells were irradiated with a dose of 2 or 8 Gy. Immediately following this, bromodeoxyuridine (BrdUr) and deoxycytidine were added to the medium to give a final concentration of 20 μM for MCF-7 BUS and TE671 and 60 μM for RT112. The deoxycitidine was added in equimolar concentration to avoid possible disturbance of the nucleotide pathway due to BrdUr.

Cells were harvested at various time points between 0 and 30 h after γ-irradiation. The samples were spun and resus-

pended in culture medium containing 10% DMSO and stored at −70°C until analysis. The control cultures were handled under identical conditions. At least two different experiments were done for each cell line and each time point was examined in duplicate within each experiment.

A pilot experiment was carried out to optimize the BrdUr concentration in these three cell lines and, in consequence, to label the DNA satisfactorily without causing cytotoxicity (10, 20, 40, 60, 80 and 100 μM). Continuous exposure to a halogenated nucleoside may impair cell proliferation directly or indirectly. In these tumour cell lines, the proliferation was not detectably affected at the BrdUr concentrations used. This observation is in good agreement with others published previously [8,29,7].

2.4. Flow cytometry

The method of Poot and Ormerod [29] was used to assess the cell cycle delays. This entails continuous labelling with 5-bromo-deoxyuridine (BrdUr) and bivariate Hoechst 33258/ethidium bromide (EB) flow cytometry. In consequence for each cytogram, the quenching of Hoechst dye allows the discrimination of chromatids according to the number of replications they underwent during the observation period and the non-quenched EB defines the different cell cycle compartments (G1, G2 and S phase).

The method used has been described previously [7]. The samples were thawed, pelleted by centrifugation, resuspended in 1-ml ice-cold staining buffer and incubated on ice in dark for 15 min. The staining buffer was 100 mM Tris (pH7.4), 154 mM NaCl, 1 mM CaCl, 0.5 mM $MgCl_2$ 0.1% (vol./vol.) Nonidet, 0.2% (wt./vol.) bovine serum albumin and 1.2 μg/ml Hoechst 33258 (final concentration). Ethidium bromide was then added to a final concentration of 2.0 μg/ml and after a further 15 min on ice the cells were analysed. Samples remained without significant deterioration for up to 8 h if stored on ice.

The flow cytometric measurements were made on an Ortho Cytofluorograph SOH or a Coulter Elite ESP as described previously by Gilligan et al. [7]. Bivariate histograms of red (EB fluorescence) vs. blue fluorescence (BrdUr-Hoechst fluorescence) were analysed and figures were prepared using the WINMDI program (supplied by Dr Joe Troter, Salk Institute, USA).

2.5. Analysis of flow cytometric data

Fig. 1 shows representative dot plots for an exponentially growing culture of TE671 cells. Initially ($t = 0$), both red and blue fluorescence gave a normal cell cycle with cells in G1, S and G2/M. Six hours after the addition of BrdUr, cells in G1 have moved into a new S phase (Sf) and incorporated

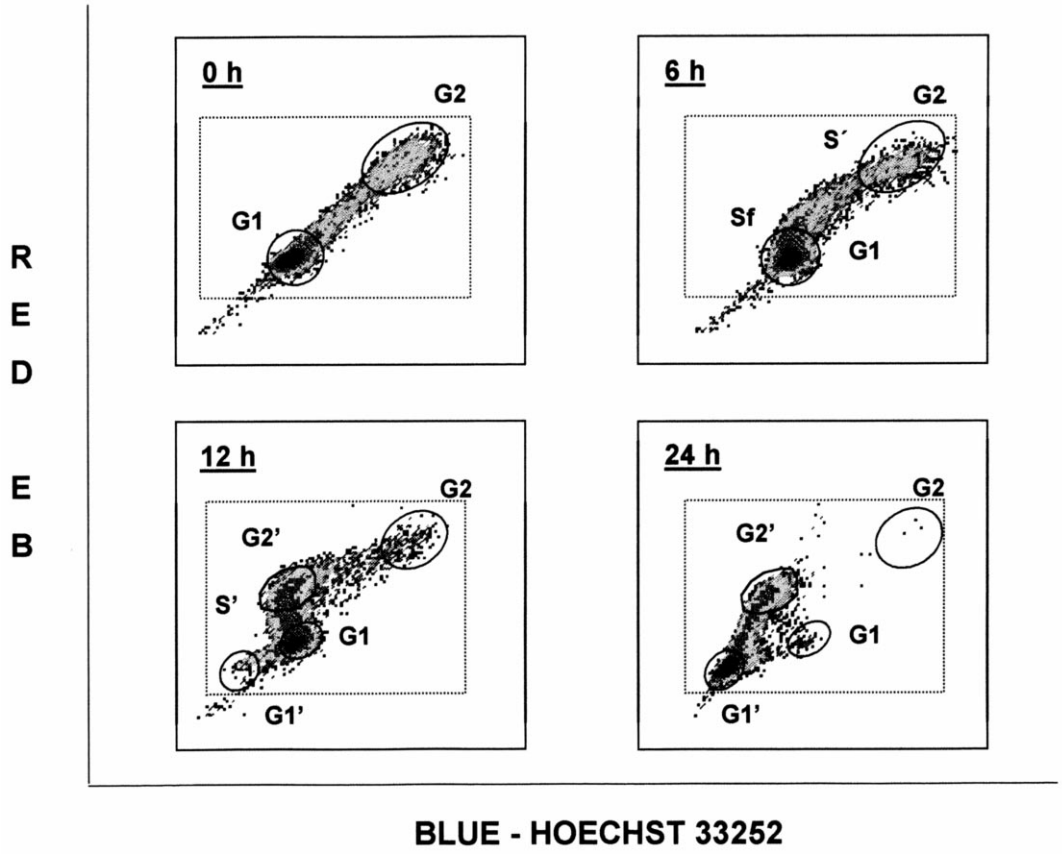

Fig. 1. Bivariate cytograms of ethidium bromide fluorescence (red) vs. BrdUr-Hoechst fluorescence (blue) for TE671 0, 6, 12 and 24 h after BrdUr supplementation.

Table 1
Clonogenic cell survival parameters after γ-irradiation

Cell line		α	β	SF2	Δ α[a]	Δ β[b]	Δ SF2[c]	Δ(α/β)[d]
TE671	w/o c	−0.36 ± 0.03	−0.02 ± 0.00	0.45 ± 0.01	1.42	0.43	0.77	3.28
	w c[e]	−0.51 ± 0.04	−0.01 ± 0.00	0.35 ± 0.03				
RT112	w/o c[f]	−0.09 ± 0.03	−0.02 ± 0.00	0.78 ± 0.04	3.07	0.09	0.74	15.78
	w/c	−0.28 ± 0.03	−0.01 ± 0.00	0.58 ± 0.02				
MCF-7 BUS	w/o c	−0.26 ± 0.02	−0.02 ± 0.00	0.55 ± 0.04	1.01	0.88	0.96	1.14
	w/c	−0.26 ± 0.02	−0.01 ± 0.00	0.53 ± 0.07				

[a] $\Delta\alpha = \alpha_{caf}/\alpha$.
[b] $\Delta\beta = \beta_{caf}/\beta$.
[c] $\Delta SF2 = SF2_{caf}/SF2$.
[d] $\Delta(\alpha/\beta = (\alpha = \beta)_{caf}/(\alpha/\beta)$.
[e] w c, treated in presence of caffeine.
[f] w/o c, treated in absence of caffeine.

BrdUr, which quenched their blue fluorescence. Cells from early and mid-S phase to G2 are labelled as S′. At 12 h cells from late S phase have reached G2 (the second G2 that is labelled as G2′) and cells moving into S phase from G1 have been labelled again as Sf. Cells starting in late S phase have divided to give a compartment labelled G1′ (the second G1). After 24 h, most of the cells have disappeared from G2 due to cell division and also many of the cells originally in G1 phase have cycled once and a new G1 (G1′) can be observed. Numerical data were obtained by drawing regions around the different cell cycle compartments (solid line in Fig. 1) and calculating the percentage of the cells in each region with respect to the all cells in the cycle (dotted line in Fig. 1).

2.6. Statistical analysis

The survival parameters obtained (surviving fraction at 2 Gy (SF2), α and β) after irradiation with and without caffeine were compared for statistically significant differences by ANOVA. The software used was GraphPad Prism (GraphPad Software Inc., San Diego, CA). Survival data were fitted to the linear-quadratic equation. The confidence intervals and the surviving fraction at 2 Gy were obtained from the experimental curve.

3. Results

3.1. Cell survival

The clonogenic cell survival parameters for the three cell lines after irradiation with or without caffeine cotreatment are shown in Table 1. Experiments were performed at least three times with each cell line, and pooled data were fitted to a linear-quadratic equation to obtain these estimates of the surviving fraction at 2 Gy, α- and β-coefficients. It can be seen that caffeine increased the sensitivity of TE671 and RT112 but it had no effect on the survival of MCF-7 BUS. This is reflected in statistically significant changes in the SF2 and α parameters for TE671 and RT112 but not in the β parameter for these lines (Table 2).

3.2. Cell cycle parameters

A full picture of the kinetic pattern of these three cell lines was obtained analysing the samples at different time intervals after irradiation. Flow cytometric dot plots display analysis of S phase (determined after BrdUr incorporation) on the *y*-axis and DNA content (by staining with EB) on the *x*-axis. Cell cycle populations were characterized as G0/G1 (2 N content with no BrdUr incorporation), S phase as variable DNA with BrdUr incorporation and G2/M as 4 N DNA content with no BrdUr incorporation. The data from the BrdUr/Hoechst-EB method have been analysed on the basis of these parameters that can be distinguished easily using this method as described previously in Fig. 1.

3.2.1. Exit from G2/M

The movement of cells from the initial G2/M phase of the cell cycle represents a measure of the delay in the passage of cells through M imposed on the cells irradiated in the G2/M phase.

Fig. 2 shows the proportion of TE671, RT112 and MCF-7 BUS cells in G2 phase at various time points from 0 to 30 h following 8 Gy (closed symbols) and control (open symbols). For TE671 and MCF-7 BUS there was a marked reduction in the rate of exit from G2 after both 2 (data not shown) and 8 Gy but there was very little delay in RT112

Table 2
Statistical differences between survival parameters in presence and absence of caffeine

Cell line	$\alpha_{caff} - \alpha$	$\beta_{caff} - \beta$	$SF2_{caff} - SF2$
	P-value		
TE671	<0.050	<0.100[a]	<0.003
RT112	<0.005	<0.100[a]	<0.005
MCF-7 BUS	<0.400[a]	<0.300[a]	<0.350[a]

[a] Not statistically significant.

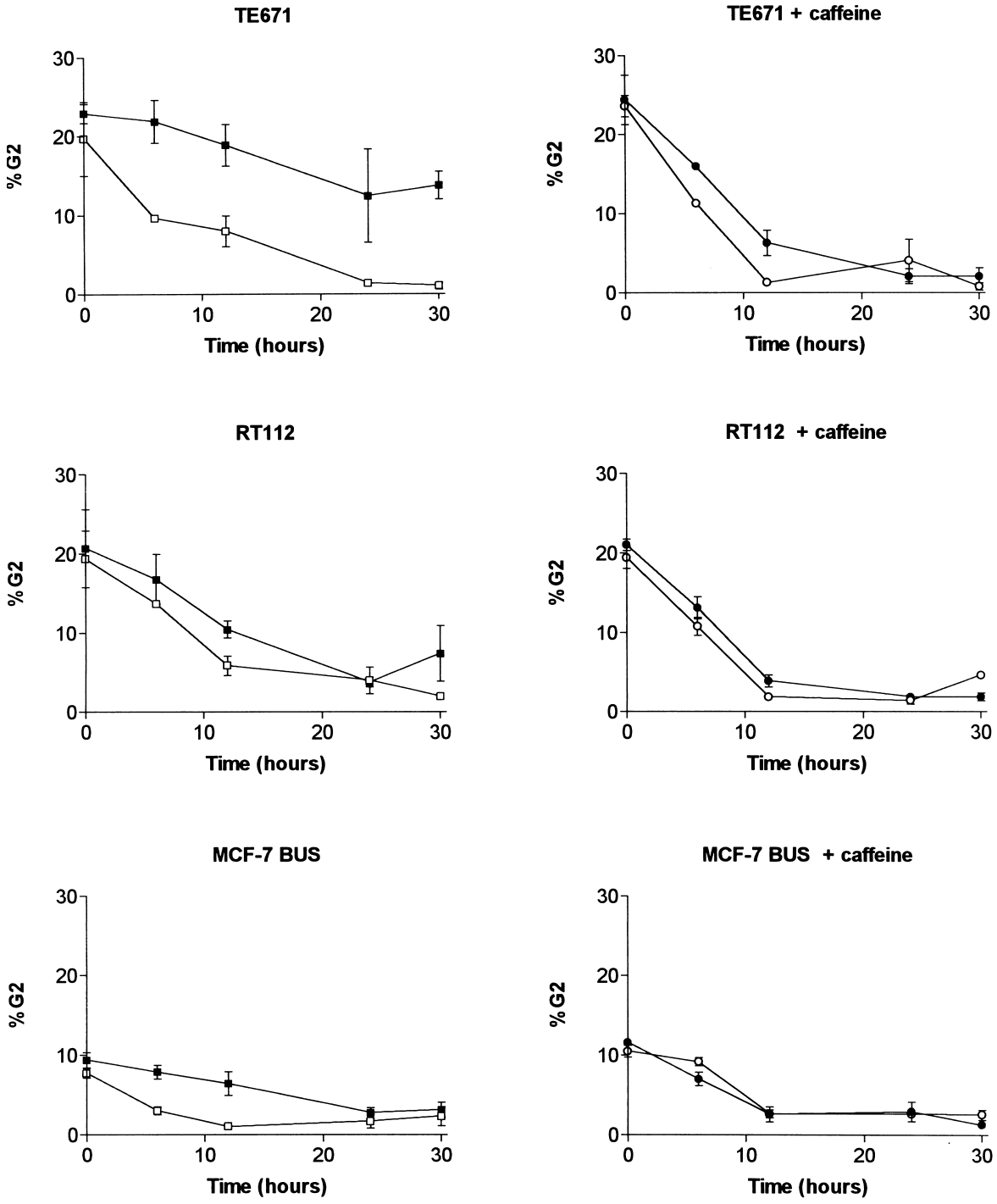

Fig. 2. Time course of cells in the first G2/M phase (% G2) treated with or without caffeine ((2 mM). Open symbols represent non-irradiated cells (control cells) and closed symbols irradiated cells (8 Gy). Points, means ± SEM of at least three independent experiments.

compared with the control values. Treated TE671 cells showed approximately 5 and 10 times more cells in G2 24 h after irradiation (2 and 8 Gy, respectively) compared with unirradiated controls. MCF-7 BUS, 1.5 and 1.2 folder 24 h after the treatment using these two doses. Caffeine treatment beginning 30 min before irradiation reduced the extent of this radiation-induced delay in TE671 and MCF-7 BUS.

3.2.2. Proportion of the cells in G1

The proportion of cells in G1 at any particular time in these experiments is a reflection of those that have moved through from G2 and those that are still in G1. Thus, an accumulation of cells in G1 is an indication of a G1 block although due account needs to be taken of the movement from G2.

Fig. 3 shows the proportion of cells in the G1 phase at various time points in controls and after 8 Gy irradiation. For TE671, there is a lower number of cells at 12 h in the treated groups (the same effect after 2 Gy, data not shown), which is totally explainable in terms of delay in cells coming through G2. Caffeine reduces the difference between treated and controls, which is again consistent with the effects in G2 being responsible for the differences in Fig. 2. There is, therefore, little evidence of a significant G1 block. There are no significant trends in the effect of radiation on RT112 in the number of cells in G1 (Fig. 3). Since there was no G2 arrest, this again indicates that there is little blockage at G1 in the treated groups. Finally, the response after γ-irradiation in MCF-7 BUS (Fig. 3) suggests a significant block in G1 after 2 and 8 Gy (1.7 and 2.15 times more cells in G1, respectively, 24 h after the treatment) due

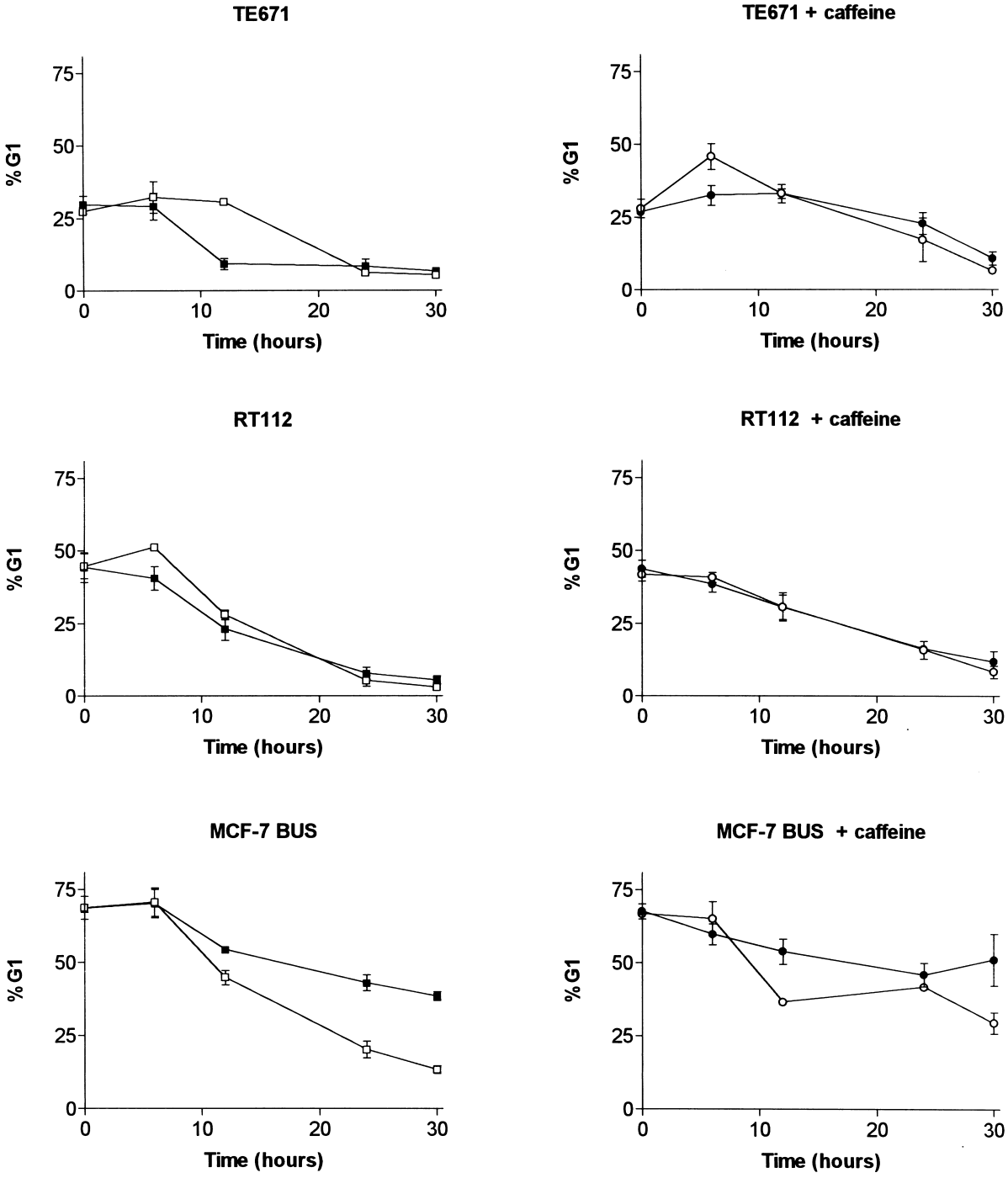

Fig. 3. Time course of cells in the first G0/G1 phase (% G1) with or without caffeine (2 mM). Open symbols represent non-irradiated cells (control cells) and closed symbols irradiated cells (8 Gy). Points, means ± SEM of at least three independent experiments.

to the number of cells in G1 in the non-caffeine treated irradiated cells remaining constant despite a reduction in entry into G1 from G2 (Fig. 2). On the other hand the non-caffeine treated unirradiated cells showed a decline in G1 even though cells were progressing into this phase from G2. In the caffeine treated group there is little difference between the irradiated and unirradiated cells, though the trend is for the unirradiated cells to leave G1 earlier. Contrasting the unirradiated cells with or without caffeine suggests that caffeine may have a slight slowing effect on cells in G1 in this cell line. Overall, therefore, there is evidence of a G1 block in MCF-7 BUS but not in TE671 or RT112.

3.2.3. Accumulation in the second G2 phase (G2′)

The accumulation of cells in the second G2 phase (G2′) is a measure of the extent of a G1 block as well as the effects of a block at G2 in cells irradiated in all phases of the cell cycle.

In RT112, radiation increased the number of cells in G2′ 10 and 24 h after 8 Gy treatment and this is somewhat reduced in the presence of caffeine (Fig. 4). In TE671, radiation does not affect the accumulation of cells in G2′ though caffeine has a small accelerating effect on this accumulation. The fact that the effect of caffeine is similar in irradiated and non-irradiated cells suggested that it is not significant. In MCF-7 BUS, the unirradiated cells increase in number in G2′ and then decrease as they move into G1′. Irradiation did not influence the rate of accumulation but did lead to a more rapid release from G2′.

3.2.4. Cells in the second G1 phase (G1′)

This parameter provides an overall measure of the cell cycle delays imposed by irradiation.

In TE671 and RT112, there is significant reduction in the rate of entry into the second G1 phase (G1′) and this is virtually eliminated by the presence of caffeine (Fig. 5). For MCF-7 BUS, the difference between irradiated and non-irradiated groups is much less but the low entry of the caffeine treated cells into G2′ makes it difficult to elucidate the effect of irradiation.

Finally a small effect of caffeine without γ-exposure was seen in RT112 (non-functional p53) and MCF-7 BUS (functional-p53). In MCF-7 BUS unirradiated cells, we observed small effects of caffeine in G1, and G1′ and G2′. In RT112 this was only seen in G2′. This difference is worthy of further investigation in order to elucidate whether this effect of caffeine is p53 related.

4. Discussion

The data here presented show a significant enhancement of radiation-induced cytotoxicity of two human tumour cell lines with non-functional p53, RT112 and TE671, when these were treated with 2 mM caffeine and irradiated in a proliferative state. The radiosensitizing effect of caffeine was seen as a significant decrease in SF2 and a significant increase in α-values whereas β-values ($P < 0.10$) remained unchanged in both of these cell lines. In RT112 the SF2 was approximately 1.4-fold, and β-fold more radiosensitive. The ratio of SF2 and -component for TE671 were 1.3 and 1.4-fold, respectively. The steepness and curvature of these survival curves can be described by the α/β ratio, and this was changed 15.8 fold for RT112 and 3.3 fold for TE671. In contrast, cells with a G1 delay after γ-irradiation (MCF-7 BUS) did not show any significant radiosensitizing effect of caffeine. These results are in good agreement with reports from Powell et al. [30], Stewart et al. [36] and Russel et al. [32] who found that cells with apparent wild-type p53 function are sensitized by caffeine to a smaller degree than those with no G1 arrest.

Inhibition of cell cycle delay has often been proposed to be the mechanism by which caffeine sensitizes cells to DNA damage [25]. Arrest at these cell cycle junctions allows an extended time for DNA repair to take place before progression through critical phases of the cell cycle: S and M phases. The arrest at the G1 checkpoint is mediated by p53-dependent induction of $p21^{waf1/cip1}$ in response to ionizing radiation [17]. The function of p53 appears to form part of a negative regulator pathway of DNA synthesis leading to G1 arrest after cellular exposure to DNA-damaging agents, since there is a close temporal association between the post-transcriptional increase in p53 protein levels and G1 arrest after irradiation [32]. In contrast, cells with mutant *p53* genes or lacking *p53* genes failed to show any increase in p53 protein after DNA damage and can result in an abnormal cell cycle response to X-rays. This correlates with a lack of G1 arrest [17,13]. Generally these cells still show a G2 arrest although there is evidence suggesting a role for p53 in the G2/M transition. The effect of p53 status on radiosensitivity, however, is inconsistent with the 'increase in repair time theory' at the G1/S boundary as p53 mutant cells are generally more radioresistant [23,34].

The G2 checkpoint seems to fit more readily with this theory as there are several lines of evidence that point towards an increase in G2 arrest being associated with an increase in radioresistance. For example, transfecting cells with *ras* and *myc* genes increases resistance and increases G2 arrest [24]. Included in this are data, which identify an abolition of G2 arrest by caffeine in parallel to changes increases in radiosensitivity. As well as suggesting an important role for G2 arrest such data also imply that the sensitizing effect of caffeine is due to its effects on cell cycle progression. Some data, however, do not fit with this and interference with repair processes has been postulated to be another mechanism by which caffeine modulates the radiosensitivity [26,11].

In relation to p53, Powell et al. [30] noted that there was a much reduced override of a G2 block in wild-type p53 cells compared with those with mutant or normal p53, so in this study we have considered whether differences in the effects

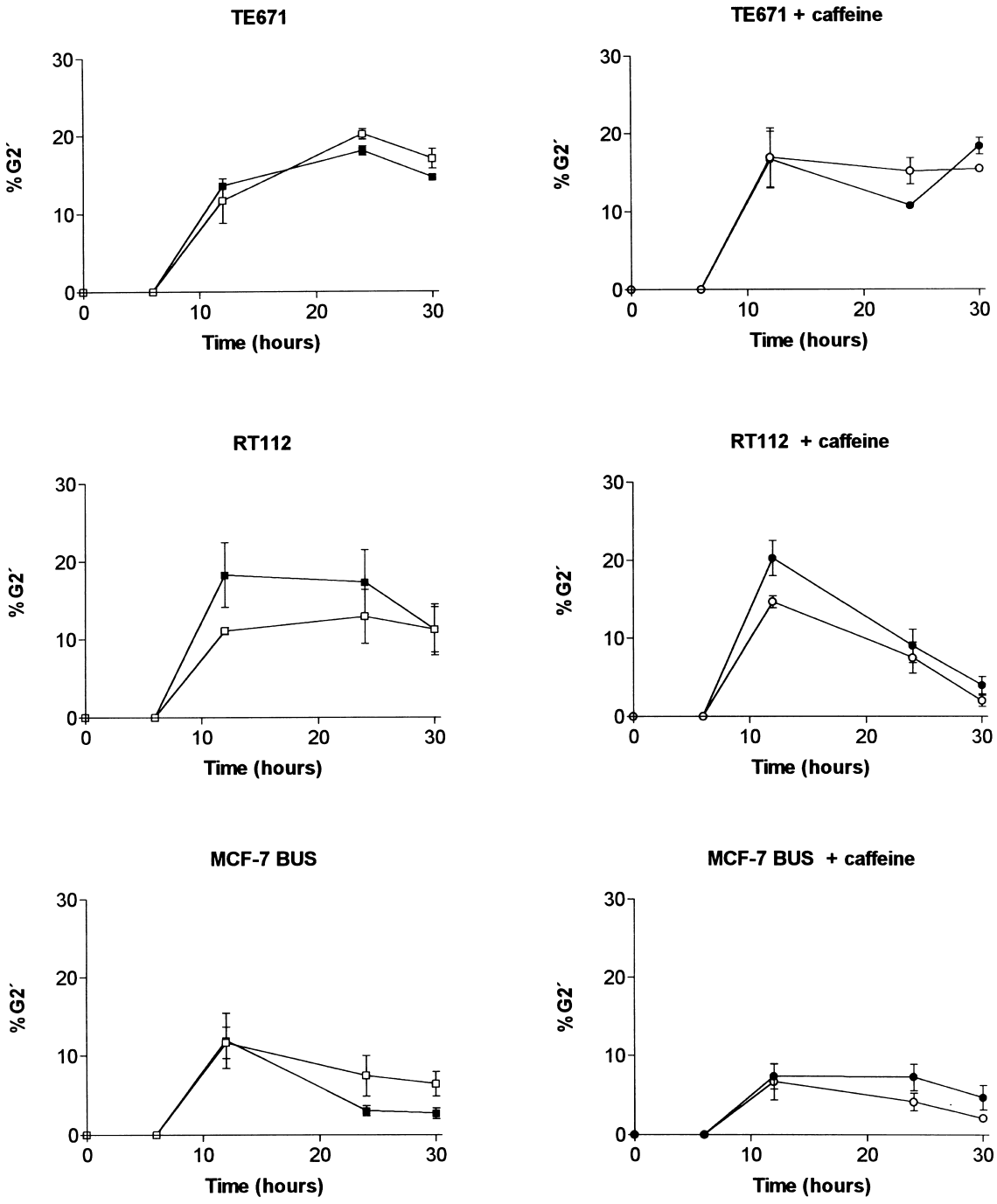

Fig. 4. Time course of cells in the second G2/M phase, G2′, (% G2′) with or without caffeine (2 mM). Open symbols represent non-irradiated cells (control cells) and closed symbols irradiated cells (8 Gy). Points, means ± SEM of at least three independent experiments.

of caffeine on cell cycle progression can explain the differences we have described, in the radiosensitizing effects of caffeine in cells with different p53 functionality.

Other DNA damage-responsive checkpoints can be observed in S and G2 phase cells. In cells that have already entered into the S phase, the induction of DNA damage can produce a rapid reduction in the rate of initiation of DNA replication within replicon clusters. Although ongoing DNA synthesis in active replicon clusters may not be stopped, inactive replicon clusters delay their initiation in response to DNA damage [14]. The delay in replicon initiation should be beneficial in allowing uninitiated replicons to be cleared of their lesions before replication. However, all those cells, which still carry out DNA lesions at the end of S phase, would be arrested in G2. In this respect, when the cells are irradiated in S, a dysfunction in the p53 status of the cells or

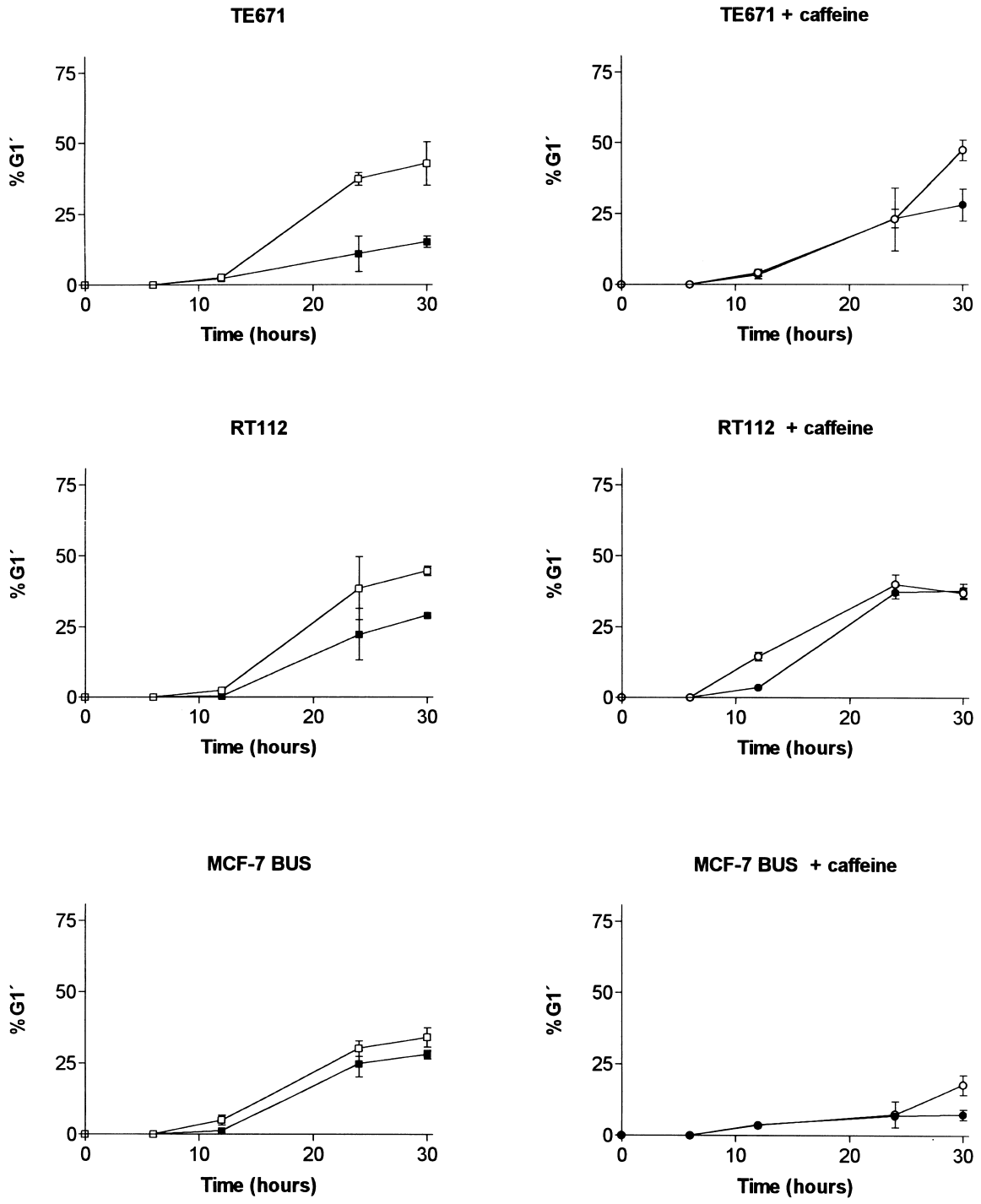

Fig. 5. Time course of cells in the second G0/G1 phase, G1′, (% G1′) with or without caffeine (2 mM). Open symbols represent non-irradiated cells (control cells) and closed symbols irradiated cells (8 Gy). Points, means ± SEM of at least three independent experiments.

a prolonged treatment with caffeine would result, at the end, in an accumulation of damaged cells in G2.

The flow cytometry technique used in this study has shown us radiation-induced cell cycle delays in three different tumour cell lines. The method has the advantage that the cells remain in their natural state and can be monitored into the cell cycle giving more information about the system kinetic. The Hoechst dye separates cells according to the number of DNA replications done. The EB resolves the cell cycle into the G1, S and G2 compartments. One limitation of this method was that some regions of interest in the cytograms might be overlapped. In this case, the cell number in G1 could be overestimated including cells from early S, and G2 could contain cells in late S. However, these

errors are cancelled because comparisons between time points for a same dose are made.

MCF-7 BUS cells have a normal p53 function and are representative of a tumour cell type that does not readily undergo p53 dependent apoptosis [33]. Here, they have been shown not to be radiosensitized by caffeine, to have a G1 arrest and to have an early G2 arrest but those cells that reached the second G2 did not arrest significantly. Irradiation did not influence the rate of accumulation but did lead to a more rapid release from $G2'$. It would be expected that irradiated cells would stay longer in $G2'$ since these cells showed a G2 block in the first G2. It is not clear why this is not the case unless, in the presence of the other blocks (both first G2 and G1) the cells that reach this stage are less damaged. Caffeine has the effect of slightly increasing the rate of decline in the $G2'$ compartment in unirradiated cells but increases the levels in irradiated cells. Again the reason for this is not clear. However, it is significant as it shows that while wt-p53 cells do not show a caffeine-reversible G2 block in cells that had been through the G1/S transition, they were capable of showing a G2 block in cells that were irradiated in G2. The implication of this is that the G1/S block does remove the importance of the G2 arrest by reducing the detectable damage when the cells reach the G2 checkpoint. In contrast to MCF-7 BUS cells, TE671 exhibited radiosensitization by caffeine, no G1 arrest, a G2 arrest in those cells irradiated in G2, no significant radiation-induced accumulation in $G2'$ but an overall delay in release from the first cell cycle, which could be abrogated by caffeine. RT112 was similar to TE671 except that the emphasis in a G2 arrest was shifted from the block in cells irradiated in G2 to those irradiated at other phases of the cell cycle.

These data stress the key role of caffeine in modifying cell cycle progression in its sensitizing effects rather than any proposed effects on DNA repair [27]. This study also emphasises the role of the G2/M transition rather than the G1/S transition in the determination of radiation-induced cell killing but that in the presence of a G1/S checkpoint cells reach G2 in a state that does not require a delay, even if they are capable of such a delay. This is stressed by the MCF-7 BUS data, which shows a G2 delay in cells irradiated in G2 but not those irradiated in G1. It is likely that those irradiated in G2 are protected by the delay but they are only present in relatively low numbers so their contribution to the overall survival level is not detectable.

The potential of pentoxifylline, a methylxanthine, to augment the effects of antitumour alkylating agents in vitro and in vivo has been examined [5,38]. In vitro pentoxifylline increased the cytotoxicity of CDDP and L-PAM. In the FSallC murine fibrosarcoma system and in the EMT6 mouse mammary adenocarcinoma, the increase in tumour cell killing was seen with CDDP, carboplatin, cyclophosphamide and thiotepa [38]. With human bladder or breast cancer xenografts in a modified subrenal capsule assay, enhancement of thiotepa effect was also observed by in vivo posttreatment with pentoxifylline. In contrast, these combinations produced no increased toxicity to normal tissues in these animals [5]. Other studies were carried out to determine whether the methylxanthines (caffeine and pentoxifylline) enhanced the cellular radiation response. Kim et al. [16] suggested that this two compounds would be considered as a radiation enhancer for clinical radiotherapy. Kelland and Steel [15] found that caffeine modified the initial slope of the acute survival curve in a human cervix carcinoma cell line. The result was a reduced survival. Wolloch et al. [40] got a similar results in an ovarian cell line that was cultured.

The data presented here confirm that p53 status can be a significant determinant of the efficacy of caffeine as radiosensitizer in this set of human tumour cell lines, and document the importance of the G2 checkpoint in this effect. This has clear significance in the potential usefulness of the G2 checkpoint in strategies for radiosensitization as it promises a differential effect in tumours with non-functional p53 and normal tissues.

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References

[1] Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 1990;249:912–915.

[2] Bhattacharjee SB, Bhattacharyya N, Chatterjeb S. Influence of caffeine on X-ray-induced killing and mutation in V7 cells. Radiat. Res. 1987;109:310–318.

[3] Bunz F, Dutnaux A, Lengauer C, et al. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 1998;282:1497–1501.

[4] Crompton NEA, Ham J, Jaussi R, Burkart W. Staurosporine and radiation induced G2 cell cycle blocks are equally released by caffeine. Radiat. Res. 1993;135:372–379.

[5] Fingert HJ, Pu AT, Chen ZY, Googe PB, Alley MC, Pardee AB. In vivo and in vitro enhanced antitumor effects by pentoxifylline in human cancer cells treated with thiotepa. Cancer Res. 1988;48:4375–4381.

[6] Friedman HS, Schold Jr SC, Varia M, Bigner DD. Chemotherapy and radiation therapy of human medulloblastoma in athymic nude mice. Cancer Res. 1983;43:3088–3093.

[7] Gilligan D, Mort C, McMillan TJ, Peacock JH, Titley J, Ormerod MG. Application of a bromodeoxyuridine-Hoechst/ethidium bromide

technique for the analysis of radiation-induced cell cycle delays in asynchronous cell populations. Int. J. Radiat. Biol. 1996;69:251–257.
[8] Ham J, Weller EM, Jung T, Burkart W. Effects of ionizing- and UV B-radiation on proteins controlling cell cycle progression in human cells: comparison of MCF-7 adenocarcinoma and the SCL-2 squasmous cell carcinoma cell line. Int. J. Radiat. Biol. 1996;70:261–271.
[9] Harris CC. Structure and function of p53 tumor-suppressor gene: clues for rational cancer-therapeutic strategies. J. Nat. Cancer Inst. 1996;88:1442–1455.
[10] Hartwell LH, Kastan MB. Cell cycle control and cancer. Science 1994;266:1821–1825.
[11] Iliakis G, Nusse M. Effects of caffeine on X-irradiated synchronous, asynchronous and plateau phase mouse ascites cells: the importance of progression through the cell cycle for caffeine enhancement of killing. Int. J. Radiat. Biol. Relat Stud. Phys. Chem. Med. 1983;43: 649–663.
[12] Kastan MB, Canman CE, Leonard CJ. p53, cell cycle control and apoptosis: implications for cancer. Cancer Metastasis Rev. 1995;14: 3–15.
[13] Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 1991;51:6304–6311.
[14] Kaufmann WK, Paules RS. DNA damage and cell cycle checkpoints. FASEB J. 1996;10:238–247.
[15] Kelland LR, Steel GG. Modification of radiation dose-rate sparing effects in a human carcinoma of the cervix cell line by inhibitors of DNA repair. Int. J. Radiat. Biol. 1988;54:229–244.
[16] Kim SH, Khil MS, Ryu S, Kim JH. Enhancement of radiation response on human carcinoma cells in culture by pentoxifylline. Int. J. Radiat. Oncol. Biol. Phys. 1993;25:61–65.
[17] Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA 1992;89:7491–7495.
[18] Lau CC, Pardee AB. Mechanism by which caffeine potentiates lethality of nitrogen mustard. Proc. Natl. Acad. Sci. USA 1982;79:2942–2946.
[19] Leeper DB, Schneiderman MH, Dewey WC. Radiation induced division delay in synchronized Chinese hamster ovary cells in monolayer culture. Radiat. Res. 1972;50:401–417.
[20] Little JB. Delayed initation of DNA synthesis in irradiated human diploid cells. Nature 1968;218:1064–1065.
[21] Martinez J, Georgoff I, Martinez J, Levine AJ. Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. Genes Dev. 1991;5:151–159.
[22] Masters JRW, Hepbunn PJ, Walker L, et al. Tissue culture models of transitional cell carcinoma: characterization of 22 human urothelial cell lines. Cancer Res. 1986;4:3630–3636.
[23] McIlwrath AJ, Vasey PA, Ross GM, Brown R. Cell cycle arrests and radiosensitivity of human tumour cell lines: dependence on wild-type p53 for radiosensitivity. Cancer Res. 1994;54:3718–3722.
[24] McKenna WG, Iliakis G, Weiss MC, Bernhard EJ, Muschel RJ. Increased G2 delay in radiation-resistant cells obtained by transformation of primary rat embryo cells with the oncogenes H-ras and v-myc. Radiat Res. 1991;125:283–287.
[25] Murnane JP. Cell cycle regulation in response to DNA damage in mammalian cells: a historical perspective. Cancer Metastasis Rev. 1995;14:17–29.
[26] Musk SR. Resuction of radiation-induced cell cycle blocks by caffeine does not necessarily lead to increased cell killing. Radiat. Res. 1991;125:262–266.
[27] Musk SR, Steel GG. Override of the radiation-induced mitotic block in human tumor cells by methylxantines and its relationship to the potentiation of cytotoxicity. Int. J. Radiat. Biol. 1990;57:1105–1112.
[28] Painter RB, Roberston JS. Effect of irradiation and theory of role of mitotic delay on the time course of labelling of HeLa S3 cells with tritiated thymidine. Radiat. Res. 1959;11:206–217.
[29] Poot M, Ormerod MG. In: Ormerod MG, editor. Analysis of cell proliferation by the bromodeoxyuridine Hoechst/ethiduium bromide. Flow cytometry. A practical approach, 2nd ed. UK: IRL Press at Oxford University Press, 1994. pp. 157–167.
[30] Powell SN, Defrank JS, Connel P, et al. Differential sensitivity of p53(−) and p53(+) cells to caffeine-induced radiosensitization and override of G2 delay. Cancer Res. 1995;55:1643–1648.
[31] Ribeiro JC, Barnetson AR, Fisher RJ, Mameghan H, Russell PJ. Relationship between radiation response and p53 status in human bladder cancer cells. Int. J. Radiat. Biol. 1997;72:11–20.
[32] Russell KJ, Wiens LW, Demers GW, Galloway DA, Plon SE, Groudine M. Abrogation of the G2 checkpoint results in differential radiosensitization of G1 checkpoint-deficient and G1 checkpoint-competent cells. Cancer Res. 1995;55:1639–1642.
[33] Siles E, Villalobos M, Jones L, et al. Apoptosis after gamma irradiation. Is it an important cell death modality? Br. J. Cancer 1998;78:1600–1606.
[34] Siles E, Villalobos M, Valenzuela MT, et al. Relationship between p53 status and radiosensitivity in human tumor cell lines. Br. J. Cancer 1996;73:581–588.
[35] Soule HD, Vazquez A, Long A, Albert S, Brennan MA. Human cell lines from a pleural effusion derived from a breast carcinoma. J. Natl. Cancer Inst. 1973;51:1409–1413.
[36] Stewart N, Hicks GG, Paraskevas F, Mowat M. Evidence for a second cell cycle block at G2/M by p53. Oncogene 1995;10:109–115.
[37] Su L-N, Little JB. Prolonged cell cycle delay in radioresistant human cell lines transfected with activated ras oncogene and/or simian virus 40T-antigen. Radiat. Res. 1993;133:73–79.
[38] Teicher BA, Holden SA, Herman TS, Epelbaum R, Pardee AB, Dezube B. Efficacy of pentoxifylline as a modulator of alkylating agent activity in vitro and in vivo. Anticancer Res. 1991;11:1555–1560.
[39] Walters R, Gurley LR, Tobey RA. Effects of caffeine on radiation induced phenomena associated with cell cycle traverse of mammalian cells. Biophys. J. 1974;14:99–118.
[40] Wolloch EH, Sevin BU, Perras JP, et al. Cell kinetic perturbations after irradiation and caffeine in the BG-1 ovarian carcinoma cell line. Gynecol. Oncol. 1991;43:29–36.