DNA repair after irradiation in glioma cells and normal human astrocytes

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We examined DNA damage responses and repair in four human glioma cell lines (A7, U87, T98G, and U373) and normal human astrocytes (NHAs) after clinically relevant radiation doses to establish whether we could identify differences among them that might suggest new approaches to selective radiosensitization. We used phosphorylation of histone H2AX visualized by immunocytochemistry to assess DNA double-strand break (DSB) formation and resolution. Fluorescence immunocytochemistry was used to visualize and quantify repair foci. Western blotting was used to quantify repair protein levels in the different cell lines before and after irradiation and during different cell cycle phases. Mitotic labeling was used to measure cell cycle parameters after irradiation. We found that the glioma cell lines repaired DSBs more slowly and less effectively than did NHAs in the clinically relevant dose range, as assessed by induction and resolution of H2AX phosphorylation, and this was most marked in the three *TP53*-mutated cell lines (T98G, A7, and U373). The glioma cells also expressed relatively high repair-protein levels compared with NHAs that were not altered by irradiation. High levels of the repair protein Rad51 in these cells persisted throughout the cell cycle, and a marked increase in Rad51 foci formation, which was not restricted to cells in G2/S phase, occurred at early time points after irradiation. *TP53*-mutated glioma cell lines demonstrated a very prominent dose-responsive G2 checkpoint and were sensitized to radiation by caffeine, which inhibits G2/S phase checkpoint activation. In conclusion, DNA repair events differed in these four glioma cell lines compared with NHAs. In particular, the three *TP53*-mutated glioma cell lines exhibited markedly increased Rad51 protein levels and marked, dose-dependent Rad51 foci formation after low radiation doses. This suggests that agents that disrupt Rad51-dependent repair or prevent G2 checkpoint activation may selectively sensitize these cells.
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Although high-grade gliomas are uncommon, these tumors account for a disproportionate loss of life years because of the almost uniformly poor prognosis. Standard local treatment includes external beam radiotherapy following maximal surgery. Attempts to improve outcome by increasing the local radiation dose have in general been unsuccessful because of necrosis in the surrounding brain, which becomes dose limiting before significantly improved local tumor control is achieved. Clearly, a more effective approach would be to develop agents that selectively sensitize these tumor cells to radiation. In the past, nitroimidazoles have been used in an attempt to sensitize the known hypoxic cell fraction, and 5-bromodeoxyuridine analogues have been added to standard-dose radiotherapy to sensitize rapidly dividing tumor cells. Neither approach has yielded clinically apparent benefit.[1,2] More recently, 2-year survival rates after surgery and radiotherapy have been improved from approximately 10% to 26% by the addition of concomitant and adjuvant chemotherapy using the alkylating agent temozolomide.[3] In vitro data, however, sug-

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gest that the combined effect of these agents is additive, with no evidence of radiosensitization.[4,5] Additional data from the same clinical study also suggest that response to radiotherapy with concomitant and adjuvant temozolomide depends on tumor O^6-methylguanine methyltransferase (MGMT) status and that around half of patients with glioblastomas will not benefit from this combination because of effective MGMT-based repair of DNA base damage.[6] This suggests that alternative strategies are necessary in patients whose tumors are resistant to DNA damage caused by temozolomide and also that additional benefit could be achieved by adding radiosensitizing agents to combined treatment regimens.

Advances in cancer cell biology and in the understanding of DNA repair have led to the design of agents that represent novel approaches to radiosensitization. Several such agents may soon become available in the clinic, including specific inhibitors of DNA repair enzymes and checkpoint proteins.[7,8] The clinical utility of these agents has been predicated on data suggesting that DNA repair may differ in tumors compared with normal tissue. Tumor cells frequently lose functionality in some aspects of DNA repair during carcinogenesis, suggesting they become more reliant on remaining functional pathways, inhibition of which may allow selectivity for tumor response. To understand how best to exploit these agents in radioresistant tumor types, it is important to investigate which repair pathways tumors utilize following clinically relevant radiation doses and which may provide selectivity compared with normal tissue.

Previous data have shown that *TP53* mutation is common in high-grade glioma.[9] This has an important influence on DNA damage responses, because TP53 signaling to effector molecules in checkpoint and apoptosis pathways is likely to be abrogated. Many reports have discussed the effect of *TP53* mutation on reducing radiosensitivity in tumor cells, although the exact mechanisms are unclear and probably vary among cell types.[10,11] *TP53* mutation represents an example of tumor-specific loss of important damage response signaling that may increase the dependence on remaining signaling events in tumor cells.

Radiation-induced cell death is mediated through induction of double-strand breaks (DSB) in DNA, which are lethal to cells if not repaired. Mammalian cells repair these lesions principally through two separate pathways: homologous recombination, which is thought to rely on the presence of an intact sister chromatid during S and G2 phase, and the more error-prone nonhomologous end joining, which utilizes the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) repair protein following end binding of Ku protein and is thought to predominate in G1 phase. Recent findings have begun to explain how the different kinetics of recruitment of these repair proteins to DNA damage sites may underlie their different contributions to cell survival.[12]

Glioma cell lines contain high levels of Rad51, which is the central protein in mediating homologous recombination repair.[13] This may be explained by aberrant TP53 function, because Rad51 protein levels are controlled at least in part by TP53 inhibitory signaling.[14,15] The potentially important role of Rad51-mediated repair in glioma cells has been confirmed in studies in which Rad51 repair foci have been visualized following damage[16] and in which Rad51 protein levels have been reduced using antisense or agents such as imatinib mesylate (Gleevec) that produce significant radiosensitization.[17,18]

The nonhomologous end-joining pathway utilizing DNA-PKcs to repair DSBs also clearly has a role in glioma cells, as demonstrated by comparing the radiosensitivity of the paired cell lines MO59J and MO59K, which are DNA-PKcs deficient and proficient, respectively.[19] Recent data have suggested that this effect may be mediated by induction of autophagy in DNA-PKcs–proficient cells.[20] In a previous study, we have also shown radiosensitization in vitro using DNA-PK inhibition to reduce nonhomologous end joining in glioma cells, but we suggested that at low damage levels the balance of repair may change in favor of homologous recombination.[21]

Relocation of repair proteins at sites of DNA DSBs is crucial in efficient repair. It has now become clear that several important repair proteins relocate specifically to sites of DSBs rapidly after irradiation. These proteins include ATM, the MRN complex, MDC1, and 53BP1.[22] Phosphorylation of histone H2AX is among the earliest changes to occur at sites of DSB damage, where it is thought to facilitate repair through maintaining structural changes in chromatin. Although it is not yet clear that H2AX phosphorylation is specific to DSBs, several groups have shown that the number of H2AX foci that can be visualized by immunofluorescence is closely related to DNA DSB induction and repair.[23–26] The appearance and resolution of these foci have therefore been used to measure DSB repair after DNA damage.

In this study, we have begun to examine in detail the response of radioresistant human glioma cell lines to clinically relevant radiation doses, with the aim of then being able to predict which novel agents known to affect DNA repair would be likely to produce clinically relevant radiosensitization. In parallel studies, we have also assessed the response of normal human astrocytes (NHAs) as a comparator normal CNS cell line, which may help indicate which repair pathways could be selectively targeted in glioma cells.

Materials and Methods

Four human high-grade glioma tumor cell lines were used in this study: A7, U87, T98G, and U373. A7, U87, and T98G cells were obtained from the European Collection of Animal Cell Cultures in October 1996. U373 cells were donated by Dr. J. Perlman in August 1995. All cell lines were confirmed *Mycoplasma* free before use. Detailed clonogenic survival data have been published previously.[27] All four cell lines are radioresistant in vitro, with survival values at 2.0 Gy of 0.7 (T98G cells), 0.67 (A7), 0.46 (U87), and 0.6 (U373). The T98G, A7, and U373 cell lines have point mutations in *TP53*; U87 is *TP53* wild type. NHAs (Clonetics Astrocyte Cell Systems) were supplied by Cambrex Bio Science (Wokingham, UK).

Immunofluorescence

Cells were grown in covered slide chambers (Labtech, Sussex, UK). Following irradiation, the cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, washed in Tween-buffered saline (TBS), and then blocked with TBS containing 0.2% Triton and 1% normal goat serum followed by TBS containing 1% normal goat serum. Primary antibody at dilutions of 1:400 (monoclonal, serine 139 phospho-H2AX; Upstate, Watford, UK), 1:1200 (polyclonal Rad51; Merck Bioscience, Hull, UK), and 1:100 (cyclin A; Vector Laboratories, Peterborough, UK) was then added and incubated at 4°C overnight. The slides were then washed with TBS/0.2% Tween and incubated with secondary antibody (Alexofluor 488 at 1:400 dilution for H2AX and 1:800 for Rad51) added for 1 h at room temperature. For dual staining, rhodamine-conjugated antibodies were also used. Slides were then washed in TBS/0.2% Tween and mounted in 4',6-diamidino-2-phenylindole. Slides were viewed with a Bio-Rad (Hempsted, UK) confocal laser microscope for dual staining by sequentially scanning the two emission channels (488 and 514 nm). For foci counting, cells were viewed under ultraviolet illumination using a Nikon inverted microscope and ×100 objective. Foci were counted in at least 100 cells per slide, with three slides counted at each dose point. Mean and SEM values of the mean for foci per cell were calculated for each dose point (JMP statistical software; SAS, Cary, NC, USA).

Mitotic Delay

We analyzed G2 delay after irradiation by examining the accumulation of cells in mitosis, identified by the immunofluorescent detection of phosphorylated histone H3 using flow cytometry. Replicate T25 cell culture flasks were seeded with 3×10^5 cells 24 h before the experiment and irradiated with 0, 0.2, 0.6, or 2 Gy X-rays at 240 tube peak potential. Immediately after irradiation, colcemid (KaryoMAX; Gibco, Paisley, UK) was added to each flask to a final concentration of 0.1 μg/ml. Two hours after irradiation, cells were harvested by trypsinization, washed in PBS, and fixed in 70% ethanol at a concentration of 1×10^6 cells/ml.

For immunofluorescence detection, cells were rinsed twice with PBS and then resuspended in 1 ml PBS containing 0.25% (vol/vol) Triton X-100. After 15 min on ice, the cells were pelleted by centrifugation and resuspended in PBS containing 1% (wt/vol) bovine serum albumin (PBS-BSA) and a 1:200 dilution of rabbit polyclonal antiphosphohistone H3 IgG (Upstate). The samples were then incubated at room temperature for 3 h, rinsed with 2 ml PBS-BSA, and centrifuged. The cell pellet was then resuspended in PBS-BSA containing a 1:30 dilution of fluorescein isothiocyanate–conjugated goat antirabbit IgG. The samples were incubated at room temperature for 30 min in the dark. After the cells were rinsed with 2 ml PBS-BSA, they were resuspended in 0.5 ml of 50 μg/ml propidium iodide in PBS and incubated for 30 min prior to flow cytometric analysis.

Caffeine Treatment for G2/S-Phase Checkpoint Inhibition

Asynchronously growing glioma cells were exposed to caffeine (2.5 mM) for 2 h prior to irradiation and for 16 h after irradiation. Mitotic delay was then measured as described above in caffeine-treated and control (medium only) cells. Surviving fractions after radiation doses of 0, 1, and 2 Gy were compared in treated and control (medium only) cells.

Results

DSB Repair Proficiency in Glioma Cells and Normal Astrocytes

We used immunofluorescence to visualize and quantify phosphorylated H2AX foci in glioma cell lines at various times after irradiation in vitro. These foci are thought to represent sites of DSBs where chromatin structural change is occurring. H2AX phosphorylation is recognized to be an early event in break repair and has a role in subsequent recruitment of repair proteins. Foci resolution is thought to occur following DSB repair, and persistent foci at late time points are thought to represent residual unrepaired DSBs. By comparing the number of foci per cell across the dose range of interest over a repair time from 30 min to 24 h, we can compare induction and resolution of DSB and therefore define DSB repair kinetics in these cell lines.

Fig. 1 shows the dose response for induction of H2AX foci in two different radioresistant glioma cell lines and NHAs, measured at 30 min after exposure. All three cell lines showed an approximately linear dose–response relationship in the dose range of 0.2–2 Gy.

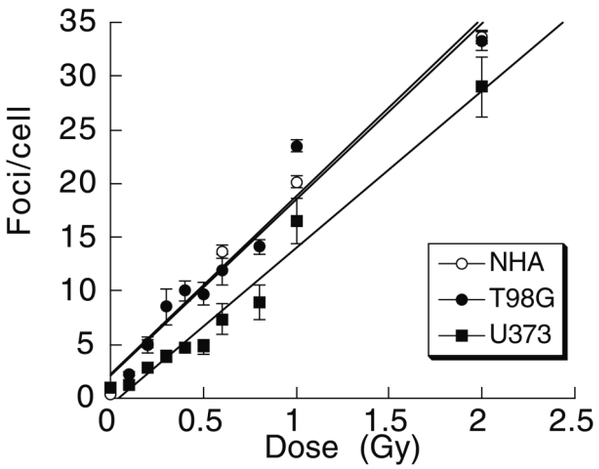

Fig. 1. Phosphorylated H2AX foci in T98G and U373 glioma cell lines and normal human astrocytes (NHA) after single radiation doses of between 0.1 and 2 Gy, measured at 30 min after exposure. Data are expressed as mean foci per cell, based on counting at least 300 cells per dose point. Error bars indicate SEM.

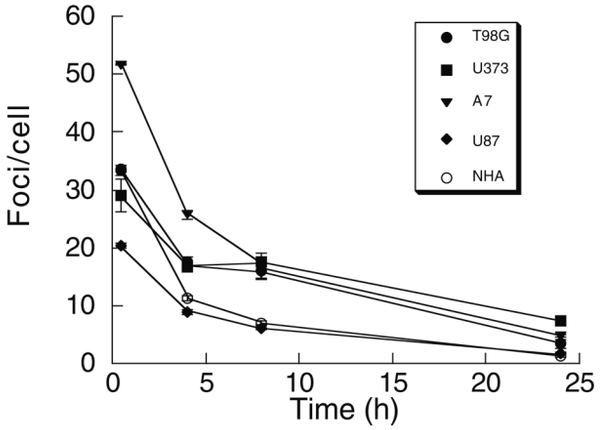

Fig. 2. Phosphorylated H2AX foci in the four glioma cell lines and in normal human astrocytes (NHA), measured at 0.5, 4, 8, and 24 h after irradiation with 2 Gy. Data are expressed as mean foci per cell, based on counting at least 300 cells per dose point. Error bars indicate SEM.

We used the same assay in the four glioma cell lines and NHAs examined at 0.5, 4, 8, and 24 h after irradiation, when resolution of foci is occurring. These data are expressed as foci resolution with time; a representation of DSB repair kinetics is shown in Fig. 2. It is notable that the observed foci per gray at early time points varied among cell lines. This may reflect differences in cell cycle distribution or nuclear morphology or subtle differences in staining. Foci resolution followed an approximately exponential curve with time in all cell lines, but the kinetics differed. In the NHAs and the U87 glioma cells (*TP53* wild type), initial resolution was rapid and foci numbers returned to background levels by 24 h. In the other three glioma cell lines (*TP53* mutant), resolution appeared to be slower, and there were residual foci apparent at 24 h. The remaining foci at 24 h are thought to represent unrepairable damage, which occurred at a rate of approximately two lesions per cell per gray. This is comparable to results found using other assays, which gave a similar estimate of residual damage at 5%–15% of initial breaks in human cell lines.[28,29]

Repair Protein Levels

Previous publications have suggested that high levels of Rad51 expression may be a common feature of tumor cell lines, including malignant gliomas, and that this may partially explain their resistance to chemotherapy and radiotherapy.[13,30] This is thought to relate at least partly to loss of inhibitory control of Rad51 expression and function by TP53.[14,15] To assess whether increased Rad51 protein expression occurs in glioma cells relative to NHAs, we compared total levels of different proteins involved in DNA repair in NHAs and in the four glioma cell lines using Western blotting before and 4 h after irradiation. We found that levels of DNA repair proteins differed between the glioma cell lines and NHAs and that these levels were not affected by 2-Gy irradiation (Fig. 3A). The glioma cell lines had higher levels of DNA-PK and, more markedly, Rad51 compared with the NHAs. Levels of Rad51 are known to be regulated in a cell cycle–dependent manner.[31] We therefore also confirmed that the increased Rad51 protein levels in the glioma cells persisted when cells of one of these lines (T98G) were held in G1/G0 phase by serum deprivation, as previously described.[27] This comparison on Western blot is shown in Fig. 3B. These data suggest that increased Rad51 protein levels are not simply due to differences in cell cycle distribution but that they may persist through the cell cycle in glioma cells and are not further increased following irradiation.

Dose-Dependent Activation of Rad51 Repair Protein

Because previous data had suggested that Rad51-mediated repair may be of particular relevance in glioma cell lines, we also examined the induction and disappearance of Rad51 foci in the same glioma cell lines.

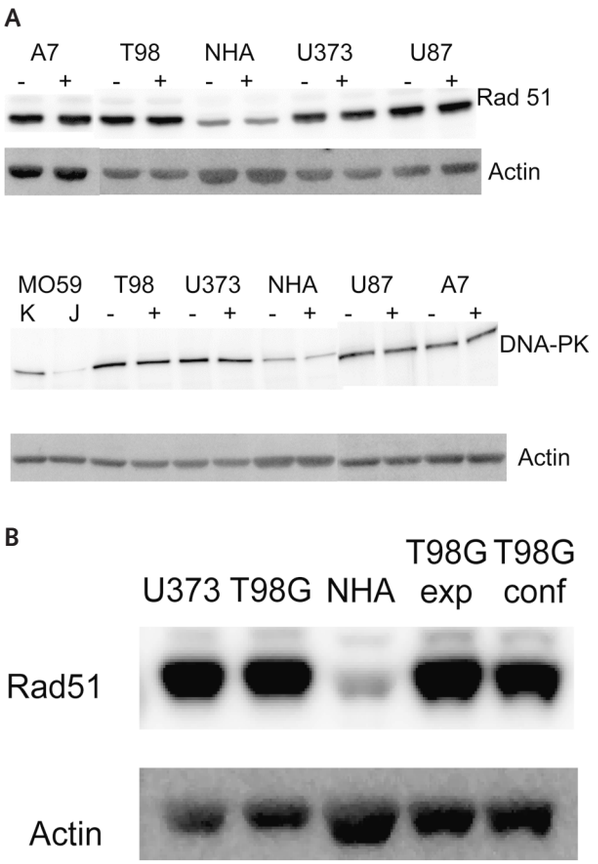

Fig. 3. Repair protein levels in glioma cells and normal human astrocytes (NHAs). (A) Levels of repair proteins Rad51 and DNA-PK assessed by Western blot in the four glioma cell lines, NHAs, and MO59J (DNA-PK deficient) and MO59K (DNA-PK proficient) cells for comparison with (+) and without (–) 2-Gy irradiation. (B) Rad51 levels in the U373 and T98G glioma cell lines, NHAs, and the T98G cell line held in confluence arrest (T98G conf) and in exponential growth (T98G exp) for comparison.

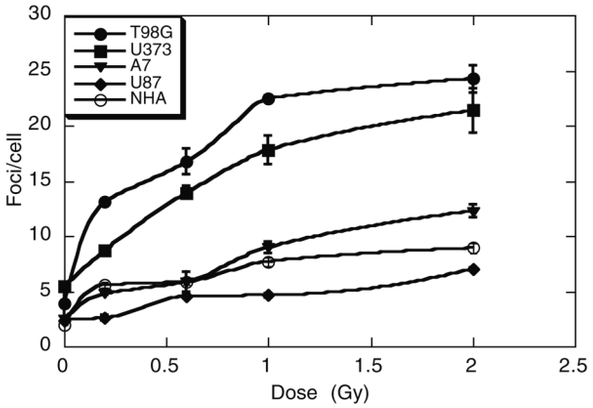

Fig. 4. Rad51 foci formation assessed in the four glioma cell lines and in normal human astrocytes (NHA) after single radiation doses of between 0.2 and 2 Gy. Data are mean foci per cell, based on counting at least 100 cells per dose point. Error bars indicate SEM.

These foci are thought to represent sites of homologous recombination at DSBs. They are known to occur in nondamaged S-phase cells, thought to be at sites of replication fork collapse, and also known to be recruited to sites of DNA damage.

Fig. 4 shows the dose response for Rad51 foci at 4 h after irradiation in the four glioma cell lines and in NHAs for comparison. It is noteworthy that the dose response was not linear over the dose range studied. High background levels are likely to indicate a high S-phase fraction with multiple sites of replication fork collapse. After irradiation, there appeared to be a marked increase in foci formation at low doses, which was most marked in T98G and U373 cells, and then a plateau effect after doses around 1 Gy. In U87 cells (*TP53* wild type) and NHAs, there was a very small increase above background levels after low doses, which was not further increased at doses greater than 1 Gy. This may be due to saturation of the effect of signaling or may represent the point at which protein levels become limiting. In all cell lines, foci numbers were reduced to background levels by 24 h (data not shown).

Cell Cycle Dependence of Repair Foci

Because the glioma cell lines demonstrated very high Rad51 foci levels, we then investigated the cell cycle dependence of these foci by co-staining with cyclin A, which is a marker for S/G2-phase cells and has been suggested to have a role in the control of Rad51 activity during different cell cycle phases after DNA damage.[32] The results for the glioma cell lines and NHAs are summarized in Fig. 5. These data suggest that, in *TP53*-mutated glioma cells (T98G, U373, and A7), Rad51 foci are commonly found in cyclin A–negative cells following irradiation and that the proportion of Rad51-positive, cyclin A–negative cells increases with dose. In NHAs and in U87 glioma cells (*TP53* wild type), Rad51 foci occurred only in cyclin A–positive S/G2-phase cells, and there was no increase in the proportion of positively stained cells with dose. These findings suggest that the high levels of Rad51 foci that we observed in the *TP53*-mutated glioma cells following low radiation doses reflect the fact that these foci increase in a dose-dependent manner throughout the cell cycle in these cells.

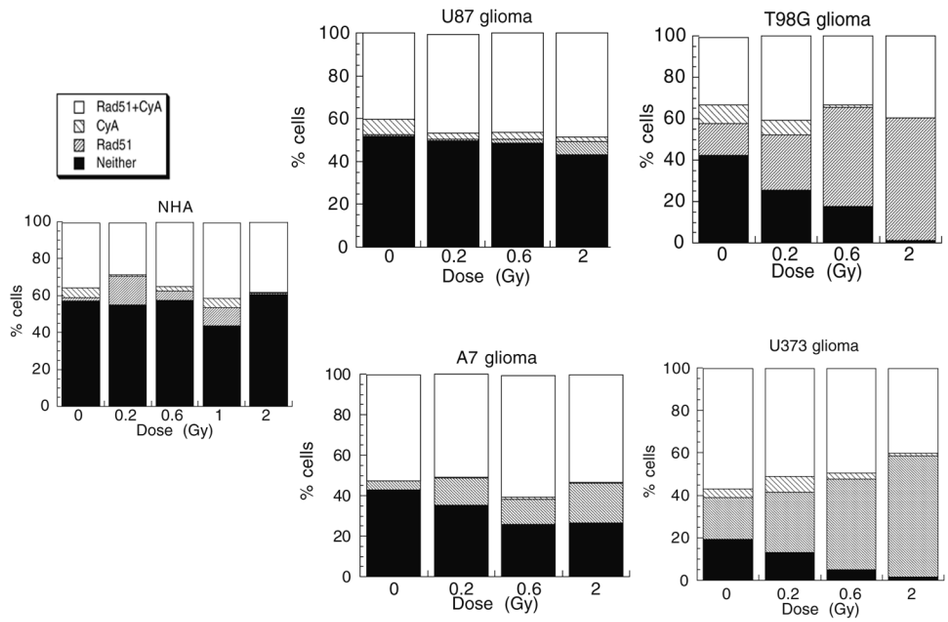

Fig. 5. Dual staining for Rad51 foci and cyclin A (CyA) in the four glioma cell lines and in normal human astrocytes (NHA), showing the proportion of cells that stained with Rad51 only (narrow hatching), cyclin A only (broad hatching), both antibodies (white), and no stain (black) as a function of radiation dose.

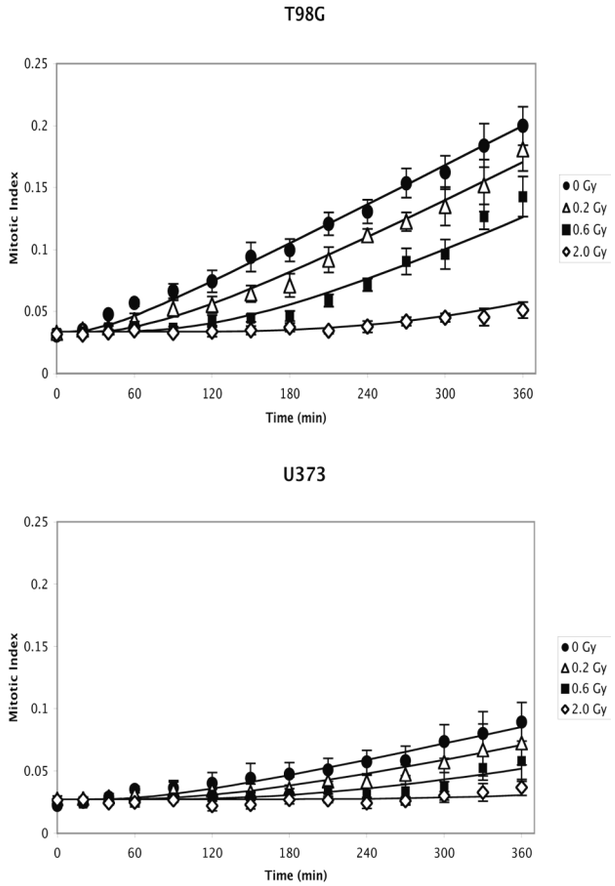

Fig. 6. Mitotic delay in T98G and U373 glioma cells after single radiation doses of between 0.2 and 2 Gy, assessed using colcemid treatment and then histone H3 immunostaining measured by flow cytometry.

Cell Cycle Checkpoint Activation

Because Rad51-mediated repair is thought to be most efficient during G2 phase of the cell cycle, we assessed the effect of low-dose irradiation on G2 delay in these glioma cells to assess how checkpoint activation in G2 may contribute to DSB repair. Fig. 6 shows the effect of doses between 0.2 and 1 Gy on mitotic delay in *TP53*-mutated T98G and U373 cells. There was a significant delay in mitotic entry in both cell lines after these very low doses, confirming a very sensitive checkpoint response in the cells irradiated during G2 phase that produced significant delays of around 3.5 h/Gy in T98G and 5.3 h/Gy in U373 cells. These findings are consistent with recent data using other *TP53*-mutated tumor cells in which DNA damage failed to induce G1 arrest but produced a significant G2/S phase delay.[33]

Cell Cycle Checkpoint Inhibition

Because the above data suggested that the *TP53*-mutated glioma cell lines may be effectively sensitized to low radiation doses by inhibiting G2/S phase checkpoints, we performed preliminary experiments using caffeine to inhibit ATM/ATR-dependent checkpoint signaling. Fig. 7A shows the effect of 2.5 mM caffeine on mitotic delay following the same radiation doses as described above in T98G cells. There was a marked reduction in mitotic delay; $D_{1/2}$ (dose to reduce mitotic index by 50%) in the control was 0.4 ± 0.064 Gy, compared with 1.39 ± 0.327 Gy in the caffeine-treated cells. The data in Fig. 7B show that this was associated with significant radiosensitization when cells were treated with caffeine in addition to radiation. This is consistent with the dependence of these cells on the G2/S-phase checkpoint following clinically relevant radiation doses.

Discussion

Although high-grade gliomas have to date resisted therapeutic endeavors aimed at improving treatment outcome, they represent one of only a small number of tumors in which local control is the main influence on outcome,

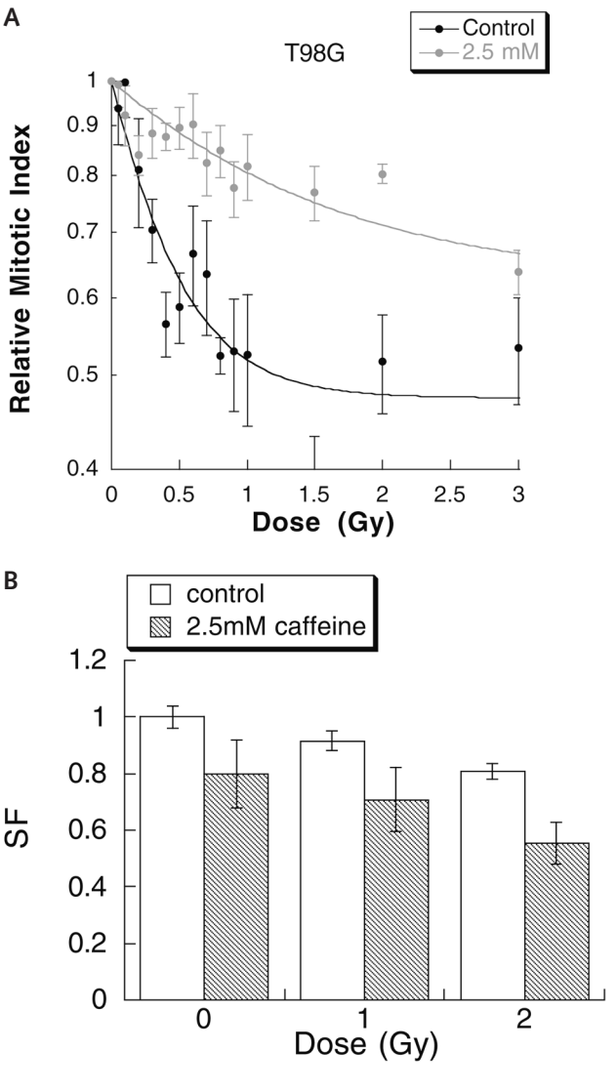

Fig. 7. The effect of 2.5 mM caffeine on mitotic delay (A) and survival after irradiation (B) in the T98G glioma cell line. Abbreviation: SF, survival fraction.

because they very rarely metastasize outside the CNS. This makes them attractive targets for radiosensitizing agents that may be used to increase local control and thereby survival. Unfortunately, historical approaches to radiosensitization have failed to achieve this goal. This may reflect problems in agent design and delivery, but these studies may have been further hampered by technical difficulties in accurate definition of tumor margins and delivery of radiotherapy fields. Technological advances in radiotherapy now permit very accurate targeting of tumor volumes within the CNS; however, significant volumes of normal tissue still need to be treated because of the infiltrative nature of these tumors. This makes gliomas an attractive model in which to test agents that may promote tumor-specific radiosensitization.

Our understanding of the molecular events that underlie repair and cellular survival following radiation treatment has increased dramatically in the last several years. It is clear that, in glioma cell lines in vitro, both currently recognized major DNA DSB repair pathways are active, but we and others have suggested that homologous recombination may predominate, particularly at low radiation doses. The data presented here lend some support to this and to the notion that high Rad51 levels may contribute to resistance to chemotherapy and radiation as well as to the genetic instability typical of these tumors. The data presented here also make it clear that the DNA repair process may differ in glioma cell lines compared with their nonmalignant counterparts in other important respects.

Our data confirmed that the induction of DSB as measured by H2AX signaling in glioma cells and NHAs occurs in a dose-dependent manner in the clinical dose range, as expected, but that foci resolution is delayed and incomplete in the *TP53*-mutated glioma cell lines. Foci resolution is not an exact measurement of repair kinetics; recent data suggest that dephosphorylation of H2AX occurs with a significant lag after DSB repair, following protein dissociation from chromatin. Interestingly, this dephosphorylation event may promote checkpoint recovery.[34] Nevertheless, the data presented here suggest that, presumably as a result of loss of function in some aspects of DNA repair, glioma cells are slower to repair and are left with more residual damage than their normal counterparts, as measured by H2AX foci resolution.

We have shown previously that, as expected, these *TP53*-mutated cells show no significant G1 checkpoint response after radiation doses of up to 2 Gy.[35] However, the data presented here demonstrate a clear dose-responsive G2 checkpoint that prolongs G2 phase by several hours even after very low radiation doses. These data imply that, because of the loss of G1 checkpoint response, the glioma cells are relatively more dependent on the G2 checkpoint to facilitate repair. Others have recently described this phenotype in other tumor cell lines and demonstrated that it predicts sensitivity to G2 checkpoint inhibition.[33]

We found that glioma cells, in common with many other tumor types, have high levels of Rad51 protein. Such high levels have been linked to high levels of homologous recombination and parallel the marked increase in Rad51 foci formation that we observed after clinically relevant radiation doses in glioma cells, which was much less marked in the NHAs. This is assumed to indicate high levels of homologous recombination, although these foci also form at stalled replication forks or by self-self assembly.[36] The finding that Rad51 foci occur outside S/G2-phase cells in the *TP53*-mutated glioma cell lines but are restricted to S/G2-phase cells in NHAs and in *TP53*–wild-type glioma cells suggests that the normal cell cycle–dependent expression of Rad51 protein and foci formation are dysregulated to some extent in these cells. This is consistent with an established role of TP53 in regulating Rad51 expression. Clearly, other processes may influence this, because both Rad51 foci numbers and the degree of dissociation with G2/S-phase cells varied among the three *TP53*-mutated cell lines that we studied. These data cannot, however, confirm whether high Rad51 expression outside G2/S phase is associated with effective repair.

Neither do these data completely explain the apparently paradoxical findings of high repair protein levels but less efficient DSB repair in gliomas compared with NHAs. However, lack of a full complement of checkpoint responses may contribute to less efficient repair of DSBs, particularly some DSB subtypes that are thought to require long repair times. It is also possible that the very high levels of Rad51 protein could inhibit repair in some circumstances, for example, by competitively binding DNA ends during G1 phase when recombination repair is likely to be inefficient because sister chromatids are not available for exchange. We plan further experiments assessing the effect of Rad51 knockdown on repair capacity in these cells to address this.

Overall, the data presented here suggest that, compared with NHAs, *TP53*-mutated high-grade glioma cell lines exhibit higher levels of recombination repair after low radiation doses. Our results also show that these cell lines utilize a very sensitive dose-dependent checkpoint in G2 phase in this dose range. This suggests that targeting Rad51-dependent repair or abrogating an efficient G2 checkpoint may effectively radiosensitize these cells and have relatively less effect on surrounding normal tissue. This has been recently established in other *TP53*-mutated tumor types using pharmacological inhibition of checkpoint Chk1.[33] The preliminary in vitro data presented here using caffeine to prevent G2/S-phase arrest provide support for this approach in gliomas.

The limitations of in vitro findings when transferred to the clinical situation are well known, and it is clear that there may be other important influences on glioma and normal CNS responses to radiation that we have not been able to address by studying isolated cell lines in culture. However, agents that target specific aspects of the DNA damage response are becoming available for use in the clinic, and data such as those presented here provide a starting point for assessing the place of these agents in preclinical studies.

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