Histone H2AX Phosphorylation as a Predictor of Radiosensitivity and Target for Radiotherapy*

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Based on the role of phosphorylation of the histone H2A variant H2AX in recruitment of DNA repair and checkpoint proteins to the sites of DNA damage, we have investigated γH2AX as a reporter of tumor radiosensitivity and a potential target to enhance the effectiveness of radiation therapy. Clinically relevant ionizing radiation (IR) doses induced similar patterns of γH2AX focus formation or immunoreactivity in radiosensitive and radioresistant human tumor cell lines and xenografted tumors. However, radiosensitive tumor cells and xenografts retained γH2AX for a greater duration than radioresistant cells and tumors. These results suggest that persistence of γH2AX after IR may predict tumor response to radiotherapy. We synthesized peptide mimics of the H2AX carboxyl-terminal tail to test whether antagonizing H2AX function affects tumor cell survival following IR. The peptides did not alter the viability of unirradiated tumor cells, but both blocked induction of γH2AX foci by IR and enhanced cell death in irradiated radioresistant tumor cells. These results suggest that H2AX is a potential molecular target to enhance the effects of radiotherapy.

Radiotherapy (RT) remains an important form of local and regional cancer therapy. Approximately 50–70% of all cancer patients receive RT during their treatment. Advances in tumor imaging and physical targeting of ionizing radiation (IR) (1, 2) and optimization of IR delivery schedules from single treatments to continuous irradiation have yielded significant improvements in patient outcome. Nonetheless, many tumors are poorly controlled by RT alone. Two major strategies to enhance the effects of IR have offered clinical benefit. The use of cytotoxic chemotherapy as a radiosensitizer (3) is based on the rationale that additional DNA damage may lower the threshold of cell death by IR. As a second approach, biological targeting (4, 5) antagonizes physiological survival mechanisms of tumors. Current biological strategies include use of angiogenesis inhibitors (6, 7), hormonal blockade (8), inhibitors of growth factor receptors (9, 10), and gene therapy (11, 12). Despite the promise of each of these strategies, our fundamental lack of understanding of molecular determinants of tumor response to IR has limited the success of all of these approaches to date.

It remains controversial whether IR-induced DNA damage mediates its tumoricidal effects predominantly via apoptosis, mitotic catastrophe, terminal differentiation, and/or other mechanisms (13, 14). Further, the well known differences between specific tumors in radiosensitivity have been ascribed to a wide range of biological effects. In particular, tissue origin, growth kinetics, and apoptosis/anti-apoptosis mechanisms (15, 16), increased DNA repair (17, 18), invasion of the extracellular matrix (19, 20), and stromal factors (21) may each play critical roles in determining tumor curability following radiotherapy. Importantly, the physiological target of IR is not DNA alone but rather DNA in the context of chromatin (22, 23) within a complex and highly regulated protein-DNA structure. Although initial tests have suggested that modulating chromatin function is highly attractive for biological targeting (24–26), deregulating chromatin function remains relatively unexplored as an approach to enhance anti-tumor effects of IR.

The nucleosome, the fundamental unit of chromatin organization in eukaryotes, consists of 200 bp of DNA wrapped around an octamer of four pairs of histones H2A, H2B, H3, and H4 (27). The histone globular domains comprise the nucleosome core, whereas the histone tails protrude, providing potential sites for protein modification such as acetylation, methylation, and phosphorylation. These histone tail modifications alter DNA accessibility and nucleosome dynamics (28), thereby affecting regulation of gene expression (29), DNA repair (30), and other nuclear events. Histone H2AX (31, 32), a minor histone H2A variant, encodes a conserved Ser-Gln-Glu (SQE) motif in the carboxyl-terminal tail. IR exposure induces rapid phosphorylation of the Ser residue (Ser139) by ATM/ATR family kinases (33–35) to form the γH2AX immunoreactive epitope at DNA double strand break sites (36, 37). γH2AX foci also form at DNA double strand breaks that arise physiologically such as during B or T cell receptor rearrangement or meiotic crossing over (38, 39).

Targeting chromatin modifications specific to DNA double strand breaks such as histone H2AX phosphorylation might enhance the cytotoxic effects of IR while circumventing adverse effects on unirradiated cells. Insofar as the phosphorylation of H2AX at DSBS has been implicated in the timely recruitment and/or retention of DNA repair and checkpoint proteins such as BRCA1 to sites of DNA damage (40–51), blocking H2AX phosphorylation may antagonize DNA repair response to IR. Indeed, H2AX-deficient mouse embryonic fibroblasts (MEFs) demonstrate defects in localization of repair factors to form...
Histone H2AX and Radiosensitivity

MATERIALS AND METHODS

Cell Culture and Xenografts—Cell lines were maintained in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal bovine serum (Intergen, Purchase, NY). Xenografts were generated by injecting SCC-61 (10^7 cells in 0.1 ml of phosphate-buffered saline) or SQ-20B (5 x 10^6 cells in 0.1 ml of phosphate-buffered saline) into the right hind limb of athymic nude mice (NCI-Frederick). Further, we have investigated H2AX phosphorylation as a potentially valuable marker for radio response and a first proof of principle for targeting chromatin modifications as a potentially valuable marker for radiotherapy outcome and examined its therapeutic potential as a biological target.

Here, we show that H2AX foci persist long after IR exposure in radiosensitive tumor cell lines but that radioreistant cell lines clear H2AX foci rapidly. Further, blocking histone H2AX phosphorylation in a radiosensitive tumor cell line enhanced IR-mediated cell death. These data provide evidence for chromatin modifications as a potentially valuable marker for radio response and a first proof of principle for targeting chromatin in radiosensitivity.

Ionizing Radiation Treatment—Clonogenic survival assays were performed as described (40). Each cell line was tested in two independent assays at each dose and/or condition. Trial to trial variability was <10%. Irradiation of tissue culture cells for detection of H2AX foci was performed on coverslips or in culture dishes using a Gammacell 1000 x-ray generator at 1.55 Gy/min. For fractionation experiments, tissues were plated at 50% confluence, irradiated with 3 or 9 Gy, returned to the tissue culture incubator for 24 h, and harvested for clonogenic assay and immunofluorescence and Western analysis. A second population was subjected to a sequence of 3 Gy, 24-h incubation, 3 Gy, 24-h incubation, and then harvest. Finally, a third population was subjected to a sequence of 3 Gy, 24-h incubation, 3 Gy, 24-h incubation, 3 Gy, 24-h incubation, and harvest. Irradiation of xenografts was performed using a Pantak (East Haven, CT) PMC 1000 x-ray generator at 1.92 Gy/min once tumors reached a volume of ~200 mm^3 (length x width x depth/2, measured using callipers). The tumorbearing limb was extended, and the body of the mouse was shielded with lead as described (41). At the indicated times, irritated animals were sacrificed, and the tumor was dissected out and immediately prepared for immunoblotting.

Immunoblotting and Immunocytochemistry—Cells or tissues (xenografts) were lysed mechanically in TEN buffer (20 mM Tris (pH 8), 150 mM NaCl, 1% Nonidet P-40), and lysates were prepared for immunoblotting with monoclonal or polyclonal H2AX antibodies (Upstate Technology, Inc., Lake Placid, NY) or with a polyclonal antibody raised against the D. melanogaster H2AX homolog carboxyl terminus (a kind gift of W. Du), incubated with secondary antibody-horseradish peroxidase conjugate, and visualized by chemiluminescence ( supersignal; Pierce). In all Western blot analyses, uniform protein loading of the SDS-PAGE was confirmed by Ponceau-S staining after transfer to nitrocellulose membrane or by immunoblotting with monoclonal anti-actin antibody (Sigma). Cells cultured on glass coverslips for 24 h were fixed in paraformaldehyde, rinsed in phosphate-buffered saline, and incubated with H2AX antibodies and then fluorescein isothiocyanate-conjugated goat anti-rabbit IgG or Alexa 540-conjugated goat anti-mouse IgG ( Molecular Probes, Inc., Eugene, OR) as secondary antibody. Slides mounted with SlowFade antifade reagent ( Molecular Probes) were imaged on an LSM510 inverted confocal microscope with a Planapochromat ×63/1.4 numerical aperture oil immersion objective (Zeiss). Before counting foci, digital images were processed with Photoshop (Adobe, San Jose, CA) to adjust brightness and contrast. Cells were judged as “positive” for H2AX foci if they displayed 10 or more discrete dots of brightness. For quantitation of foci, a minimum of 300 cells were analyzed for each time point. All data points represent mean ± S.D. of three experiments. BRCA1 polyclonal antibodies were obtained from Oncogene Research Products (San Diego, CA).

Peptide Transduction—Peptides corresponding to human H2AX residues 113–121 (underlined) in their unphosphorylated ( Ac-CKATQASQEY-NH2, Unphos P) and phosphorylated (Ac-CKATQASPO2S(PO4)QEY-NH2, Phos P) forms were synthesized and puri-
Histone H2AX and Radiosensitivity

**RESULTS**

**H2AX Phosphorylation in Human Tumor Cells**—We used radiosensitive SQ-20B and radiosensitive SCC-61 human squamous cell carcinoma cell lines (55) to determine the relationship between radiation dose, survival, and \(\gamma\)H2AX focus formation over the clinically relevant dose range from 0 to 10 Gy. First, we confirmed the relative radiosensitivity of SQ-20B and SCC-61 by comparing apoptosis as measured by loss of propidium iodide (PI) exclusion and clonogenic survival by a colony formation assay. Within 24 h after a 3-Gy dose, 95% of SQ-20B and 100% of SCC-61 cells exhibited \(\gamma\)H2AX foci. Foci were observed in 100% of both cell types at 5 and 10 Gy. Similarly, total \(\gamma\)H2AX immunoreactivity (Fig. 1e) also increased with IR dose in both SQ-20B and SCC-61 cells. These data show that induction of \(\gamma\)H2AX foci is IR dose-dependent and suggest that \(\gamma\)H2AX is an indicator of clonogenic survival and induction of apoptosis.

We next examined the kinetics of \(\gamma\)H2AX phosphorylation in SQ-20B and SCC-61 cells after the clinically relevant dose of 3 Gy. At this dose, 18% of SQ-20B and 6% of SCC-61 retain viability by clonogenic assay. 30 min after 3 Gy, 98% of SQ-20B and 100% of SCC-61 cells retained \(\gamma\)H2AX foci, but thereafter the number of cells with foci decreased (Fig. 2a). At 1 h, foci remained in 76% of SQ-20B cells and 96% of SCC-61 cells. At 6 h, foci persisted in only 40% of SQ-20B but were retained in 80% of SCC-61 (\(p < 0.001\)). After 24 h, the number of SQ-20B cells with foci dropped to 10%, whereas SCC-61 remained at 41%. Linear regression analysis yields distinct half-times for clearance of \(\gamma\)H2AX in the two cell lines, 2.6 h (\(R^2 = 0.996\)) for SQ-20B and 14.6 h (\(R^2 = 0.920\)) for SCC-61. Parallel kinetics of \(\gamma\)H2AX clearance were observed for the number of \(\gamma\)H2AX foci per cell (Fig. 2b).

To further investigate the relationship between \(\gamma\)H2AX and radiosensitivity in human tumor cells, we studied induction and loss of \(\gamma\)H2AX from SCC-12V and SCC-12 F.2a, two cell lines isolated from a single human squamous cell tumor, which demonstrate distinct survival following radiation as measured by apoptosis assays (Fig. 2c) and clonogenic survival (56). 3 Gy induced \(\gamma\)H2AX foci to a similar extent in each cell line, but the kinetics of \(\gamma\)H2AX focus removal were distinct (Fig. 2d). By 6 h after IR, only 20% of the relatively radiosensitive SCC-12V cells exhibited \(\gamma\)H2AX foci, whereas 85% of SCC-12 F.2a retained foci. By 24 h, almost all SCC-12V cells were free of foci.
whereas 40% of SCC-12 F.2a remained γH2AX-positive (p < 0.001). We next determined γH2AX kinetics in DU-145 and PC-3, two human prostate adenocarcinoma cell lines that differ in radiosensitivity (57). 3 Gy induced γH2AX in 93% of DU-145 and 99% of PC-3 cells at 1 h. By 24 h, 12% of radiosensitive DU-145 cells remained γH2AX-positive compared with 50% of radiosensitive PC-3 cells (Fig. 2d, p < 0.001). Loss of γH2AX foci might represent H2AX dephosphorylation and/or mobilization from chromatin. To assay total phosphorylated H2AX, SQ-20B and SCC-61 cell extracts obtained at 24 h after a single 9 Gy dose were analyzed by Western analysis (Fig. 2e). Phosphorylated H2AX immunoreactivity declined markedly by 24 h, consistent with loss of γH2AX foci. However, total H2AX protein abundance did not change appreciably in the period following IR (data not shown).

Compared with SCC-61, SQ-20B also displays radioresistance in vivo, based on differential regression and regrowth of xenograft tumors after IR. Thus, we examined γH2AX kinetics after irradiation of 200 mm3 xenograft tumors in athymic nude mice. Tumor tissue was excised 0.5, 1, and 24 h after 3 Gy, and extracts were analyzed by Western analysis (Fig. 2f). Irradiation induced γH2AX in both SQ-20B and SCC-61 xenografts. By 24 h, immunoreactivity was lost from SQ-20B tumors but remained detectable in the SCC-61 tumors. Taken together, these results indicate that the rate of clearance of γH2AX foci and/or immunoreactivity correlates with the radiosensitivity of human tumor cells and xenografts.

γH2AX Kinetics after Fractionated Radiation—To investigate the effect of sequential doses of IR on γH2AX kinetics in a model for fractionated radiotherapy, we compared γH2AX accumulation in SQ-20B and SCC-61 cells treated with a single 9-Gy dose or three fractions (divided doses) of 3 Gy at 24-h intervals. After a single 9-Gy dose, 0.37% of SQ-20B and 0.07% of SCC-61 cells form colonies in a clonogenic survival assay (Fig. 3a). Treatment of SQ-20B or SCC-61 cells with 9 Gy induced foci in 100% of cells, consistent with prior results (e.g., see Fig. 1c). Subsequently, the fraction of cells displaying foci slowly decreased. At 24 h, 28% of SQ-20B cells and 96% of SCC-61 retained γH2AX foci (Fig. 3b). By 72 h after 9 Gy, γH2AX foci persisted in 18% of SQ-20B and 45% of SCC-61 cells.

Dividing 9 Gy into three equal doses delivered at 0, 24, and 48 h further enhanced the differences in clonogenic survival, apoptosis and persistence of γH2AX foci between SQ-20B and SCC-61 (Fig. 3, a and b). The initial 3 Gy dose decreased SQ-20B survival to 18%. However, the second and third 3 Gy doses had proportionately less effect on survival, yielding surviving fractions of 9.3 and 7.0%, respectively. A similar pattern was observed in the PI assay of apoptosis (data not shown). In SQ-20B cells, γH2AX foci disappeared with similar kinetics after each 3-Gy dose, so that by 24 h, <20% of cells remained γH2AX-positive. By contrast, SCC-61 cell survival was markedly decreased by each successive dose, so that 5.8% survived after the first 3 Gy, and only 0.9 and 0.2% survived the second and third doses. Unlike SQ-20B, the proportion of γH2AX-positive SCC-61 cells increased over the 72-h time course. At 24 h after the third 3 Gy fraction, 58% of cells retained foci. The size and intensity of foci in SCC-61 cells also increased during the time course (data not shown). Western analysis of SQ-20B and SCC-61 cell lysates revealed similar levels of γH2AX immunoreactivity 48 h after the single 9 Gy dose (Fig. 3c). In the fractionation model, a distinct pattern was observed. Whereas SQ-20B cells display a only residual γH2AX immunoreactivity 24 h after the second or third 3 Gy doses, γH2AX accumulated after each dose in the SCC-61 cells. By 24 h after the third dose, total γH2AX in SCC-61 was considerably greater than in SQ-20B.

H2AX Tail Peptides Inhibit γH2AX and BRCA1 Foci—Based on H2AX−/− mice models (52, 53), we hypothesized that blocking the pathway from DNA damage to H2AX phosphorylation might slow recruitment and activation of repair and checkpoint proteins, thereby sensitizing human tumor cells to IR. As an initial approach, we designed peptide mimics of the unphosphorylated (UnphosP, Ac-CATQASQY-NEH2) and phosphorylated (PhosP, Ac-CATQASQY-NEH2) forms of the H2AX carboxyl-terminal tail as competitors of H2AX for phosphorylation by upstream kinases or binding to downstream targets. To validate this approach, we transduced (58) H2AX tail and control peptides into MEFs and tested the cells for response to IR. To confirm that both UnphosP and PhosP peptides could be introduced into cells, the peptides were labeled with a cysteine-reactive fluorescent tag and transduced. Under these conditions, nearly 100% of MEFs demonstrated

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* M. A. Beckett and R. R. Weichselbaum, unpublished results.
bright nuclear and cytosolic fluorescence both before and after irradiation with 10 Gy (Fig. 4a). When cells were transduced with vehicle alone or with a nonfluorescent control peptide and irradiated with 10 Gy, the MEFs became strongly γH2AX-positive (Fig. 4b). However, transducing MEFs with either H2AX tail peptide prior to the 10-Gy dose blocked the formation of γH2AX foci (Fig. 4b). By contrast, cell killing by IR was increased following transduction of MEFs with the tail peptides. Transduction with either H2AX tail peptide decreased clonogenic survival after 10 Gy compared with MEFs mock-transduced with vehicle alone (UnphosP, p < 0.001; PhosP, p < 0.001) (Fig. 4c). Recently, H2AX has been shown to mediate
recruitment of “later” appearing DNA repair proteins to double stand breaks (43, 46). To investigate whether the H2AX tail peptides may block the recruitment of a representative “late” DNA repair protein to presumptive DNA double strand breaks, we examined the subcellular localization of BRCA1 in MEFs treated with 10 Gy (Fig. 4). BRCA1 subnuclear foci formed normally in irradiated controls but were markedly reduced in MEFs transduced with either of the UnphosP and PhosP H2AX tail peptides. Taken together, these data suggest that H2AX tail peptides may enhance cell death by inhibiting normal DNA repair and/or checkpoint responses.

To extend these findings, we transduced the tail peptides into SQ-20B and SCC-61 human tumor cells. Both the UnphosP and PhosP H2AX tail peptides effectively blocked accumulation of γH2AX foci after 3 Gy (Fig. 5a). Importantly, the H2AX tail peptides significantly increased radiation-induced apoptosis in both the SQ-20B and SCC-61 cells (Fig. 5, 6 and c). However, the relative induction of apoptosis in SQ-20B cells following all IR doses studied was significantly greater than in SCC-61 cells. Interestingly, the clonogenic survival of radioresistant SQ-20B cells following 3 Gy was inhibited by both H2AX tail peptides (UnphosP, p < 0.001; PhosP, p < 0.001), yet the radiosensitive SCC-61 cells displayed a minimal further loss of clonogenic survival after 3 Gy when treated with H2AX peptides.

DISCUSSION

Whereas conventional radiotherapy protocols deliver identical amounts of IR to patients, the clinical outcomes can be dramatically different between tumor types. Clearly, the difference in curability among tumors can be partly attributed to distinct DNA damage responses. Without a clear understanding of the mechanism of radioresistance, precise methods to predict and modulate tumor response remain elusive. As an initial approach, we studied the kinetics of γH2AX induction by clinically relevant doses as a potential marker of tumor radioresistance. We found a correlation between radiosensitivity and the kinetics of γH2AX clearance in human tumor cell lines and xenografted tumor models. SQ-20B, a cell line derived from a head and neck cancer unsuccessfully treated with radiotherapy, cleared γH2AX foci with a shorter half-life than SCC-61, a matched radiosensitive tumor cell line. A similar pattern was observed in two other pairs of matched radioresistant and
radiosensitive human tumor cell lines.

Interestingly, by 24 h after a single 3 Gy dose, radiosensitive cell lines had largely cleared γH2AX foci, whereas the radiosensitive cells remained γH2AX-positive. We were curious whether the residual γH2AX might suggest persistent unrepaired DNA damage. Consistent with this model, the delivery of multiple 3 Gy doses at 24-h intervals magnified both the differences in γH2AX removal and loss of viability between the radiosensitive and radiosensitive tumor cells. It is relevant to note that similar time intervals and doses are used clinically in RT, suggesting γH2AX clearance as a predictive indicator for efficacy of therapy. Immunoassay of γH2AX status 24 h after RT might provide a predictor of tumor radiosensitivity early in therapy. Monitoring γH2AX accumulation by serial biopsy might permit changes in radiation dose and/or delivery individualized to each patient’s tumor.

The apparent association between γH2AX and cell survival suggested a model whereby H2AX phosphorylation may compete with endogenous H2AX for phosphorylation by ATM/ATR family kinases and/or for binding to DNA repair and checkpoint proteins. Alternatively, the peptides may block the accumulation of a sufficient concentration of DNA repair proteins at DSBs for optimal repair of potentially lethal damage.

A similar pattern was observed in the human tumor cell lines. Here, the response of the radiosensitive cell line SQ-20B was particularly striking. The H2AX peptides led to a >2-fold increase in IR-induced apoptosis at doses from 1 to 10 Gy. In turn, a clonogenic assay of cell survival after 3 Gy with or without H2AX peptides revealed a proportional loss of viability. SCC-61 tumor cells, which have a higher apoptotic response to IR alone, were less affected by the peptide treatment in both assays. Whereas experimental tumors that display a high apoptotic index after IR demonstrate greater radiation response, the relative contribution of apoptosis to the cure of human tumors remains under investigation (13, 14). Nonetheless, modulating IR-induced apoptosis via H2AX tail peptides might prove a successful treatment strategy for radiosensitive tumors.

Our results demonstrate proof of the principle that H2AX antagonists may be useful in radiotherapy. Unlike the well-known drawbacks of cytotoxic agents used in chemo-radiation (59), targeting H2AX may have no deleterious effects on unirradiated tissue. Since DNA double strand breaks can be limited to the tumor via physical targeting of radiation, biological targeting with H2AX antagonists may provide a route to localized radiosensitization.

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