Cell cycle checkpoint signaling involved in histone deacetylase inhibition and radiation-induced cell death

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Abstract

In breast cancer, radiation has a central role in the treatment of brain metastasis, although tumor sensitivity might be limited. The tumor cell defense response to ionizing radiation involves activation of cell cycle checkpoint signaling. Histone deacetylase (HDAC) inhibitors, agents that cause hyperacetylation of histone proteins and thereby aberrations in the chromatin structure, may also override the DNA damage defense response and facilitate the radiation-induced mitotic cell death. In experimental metastasis models, the human breast carcinoma cell line MA-11 invariably disseminates to the central nervous system. We compared profiles of *in vitro* MA-11 cell cycle response to ionizing radiation and HDAC inhibition. After radiation exposure, the G_2-M phase accumulation and the preceding repression of the G_2 phase regulatory factors Polo-like kinase-1 and cyclin B1 required intact G_2 checkpoint signaling through the checkpoint kinase CHK1, whereas the similar phenotypic changes observed with HDAC inhibition did not. MA-11 cells did not show radiation-induced expression of the G_1 cell cycle inhibitor p21, indicative of a defective G_1 checkpoint and consistent with a point mutation detected in the tumor suppressor *TP53* gene. Increase in the p21 level, however, was observed with HDAC inhibition. Following pretreatment with the HDAC inhibitor, the efficiency of clonogenic regrowth after irradiation was reduced, which is in accordance with the concept of increased probability of mitotic cell death when the chromatin structure is disrupted. Among molecular cell cycle–targeted drugs currently in the pipeline for testing in early-phase clinical trials, HDAC inhibitors may have therapeutic potential as radiosensitizers. [Mol Cancer Ther 2005;4(8):1231–8]

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Introduction

In breast cancer, radiation as a therapeutic modality has well-documented palliation effects on advanced metastatic disease of the brain or meninges. Within this patient population, however, survival may vary from a few months to a couple of years. Hence, improvement of the standard therapy might potentially benefit many patients (1).

We have previously characterized the MA-11 human breast carcinoma cell line for its ability to form experimental organ-specific metastases *in vivo*. This cell line was established from micrometastatic cells enriched from a bone marrow sample taken from a patient who was clinically devoid of metastatic disease (2); however, it was unexpectedly found to form metastases within the central nervous system after systemic injection in rodents (2–4). This experimental metastasis model has been characterized for therapeutic responses to a variety of pharmacologic compounds with cytotoxic activity (3, 5).

Cell cycle checkpoints constitute regulatory mechanisms that do not allow a new phase of the cell cycle to proceed before the previous one is completed (6). The tumor cell response to DNA damage involves a temporary cell cycle delay at the G_1-S or G_2-M boundaries, to activate a cascade of responses to the damage, ultimately leading to cell survival if the DNA is properly repaired, or, if not, to apoptotic or mitotic cell death (7).

The tumor suppressor protein p53 is the primary regulator of the G_1 checkpoint (7). Essentially, in tumor cells with intact p53 function, DNA damage leads to rapid p53 stabilization by posttranslational protein modifications as well as induction of the G_1 phase inhibitor p21 (7).

In the normal dividing cell, the transition from the G_2 phase to mitosis is inhibited through phosphorylations of the Cdc2 kinase of the Cdc2/cyclin B complex. On the onset of mitosis, these inhibitory phosphorylations are removed by the Cdc25C phosphatase. The activation of Cdc25C requires positive regulatory phosphorylation, accomplished by the Polo-like kinase-1 (Plk1; ref. 8). DNA damage–induced G_2 checkpoint signaling, initiated by the ATM kinase and communicated through downstream mediator proteins like p53 and the checkpoint kinase CHK1 (6, 9), will ultimately disrupt the interaction of Cdc25C with Cdc2 (6). We have previously found that the mechanism of the G_2 phase response to ionizing radiation comprises repression of the genes for Plk1 and cyclin B1, *PLK* and *CCNB1* (10).

Molecular targeted agents can have direct effects on the cellular response pathways implicated on exposure to ionizing radiation (11). A variety of pharmacologic compounds, designed to target cell cycle regulatory mechanisms, have been shown to override the DNA damage defense response that prevents mitotic entry (9, 11). Hence, such agents may have a therapeutic potential as radiosensitizers by facilitating mitotic cell death, and several are currently tested in early-phase clinical trials. We have recently reported that pharmacologic inhibition of the CHK1 kinase counteracted the tumor cell defense responses on *PLK* and *CCNB1*, and thereby the G_2 arrest, following radiation exposure (10, 12). In agreement with this, the concomitant treatment with the CHK1 inhibitor seemed to amplify the cytotoxic effect of ionizing radiation on clonogenic regrowth (12).

Drugs that modify the cellular chromatin structure may also radiosensitize tumor cells. Taxanes, which disrupt chromatin structure and chromosome segregation in mitosis, are currently used clinically as radiosensitizers in the treatment of non–small-cell lung cancer and head-and-neck cancer (13). Cellular treatment with histone deacetylase (HDAC) inhibitors causes hyperacetylation of histone proteins, which leads to aberration in the chromatin structure (14). In addition to this, the perturbation by HDAC inhibitors of cell cycle checkpoint signaling (15) might constitute the cellular mechanism by which these compounds enhance tumor cell sensitivity to radiation treatment.

Currently, several HDAC inhibitors are undergoing early-phase clinical investigation (14). Such pipeline drugs are not easily accessible, and in this report we have used a commercially available HDAC inhibitor, trichostatin A (TSA). TSA has shown excessive toxicity under *in vivo* conditions, and in this study we have therefore compared the biological mechanisms involved in the responses of the MA-11 cell cycle phenotype after exposure to TSA and ionizing radiation in cultured cells. A reduction in MA-11 clonogenic regrowth by cotreatment with TSA and radiation is further indicated.

Materials and Methods

Cell Lines and Experimental Treatments

The human carcinoma cell lines MA-11 and MT-1 were cultured as previously described (4). High-energy radiation from a ^{60}Co source was delivered at a rate of ~1 Gy/min. The unirradiated control cells were simultaneously kept at room temperature to obtain comparable conditions. The commercially available HDAC inhibitor TSA (Sigma-Aldrich Norway, Oslo, Norway) was added to the media at final concentrations of 10 to 300 nmol/L. In experiments using the selective CHK1 kinase inhibitor UCN-01 (National Cancer Institute, Bethesda, MD), the compound was added to the cell media at a final concentration of 100 nmol/L, as recommended by the supplier, 15 minutes before irradiation or TSA treatment.

Western Blot Analysis

Protein expression was measured by means of standard Western blot technique, essentially as previously described (10). The membranes were stained with amidoblack to evaluate equal protein loading, and subsequently hybridized with primary antibodies: anti–acetyl-histone H4 (Upstate, Lake Placid, NY), anti–α-tubulin (Calbiochem, Nottingham, United Kingdom), anti–poly(ADP-ribose) polymerase-1 (Calbiochem), anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-MDM2 (Chemicon International, Temecula, CA), anti-Plk1 (Zymed Laboratories, Inc., San Francisco, CA), and anti-p21 (Santa Cruz Biotechnology).

Flow Cytometry Analysis

MA-11 cells were harvested in ice-cold PBS, and after centrifugation the cell pellets were fixed in 100% methanol. To determine the fractions of cells in the G_1, S, and G_2-M phases from the cell cycle distribution, the cells were stained with 1.5 μg/mL Hoechst 33258 in PBS and analyzed in a FACStar+ flow cytometer (Becton Dickinson, San Jose, CA), as previously described (10).

Mutation Analysis of the *TP53* Gene

DNA extracted from the cell lines was analyzed for possible *TP53* mutations using the method of constant denaturant gel electrophoresis (16). The screening was done using PCR to amplify exons 2, 3, and 6 to 11 individually, as well as the large exons 4 and 5 each as two sequential PCR products (17). The sample that showed aberrantly migrating bands, indicating mutation, was reamplified using the original set of primers, of which one was biotinylated, and the same thermal cycling condition. The biotinylated PCR product was directly sequenced by means of the standard dideoxy method and Dynabeads M280-streptavidin (Dynal, Oslo, Norway) as solid support.

Northern Blot Analysis

Expression of RNA was measured by means of standard Northern blot technique, as previously described (10). The human cDNA probe for *TP53* was provided by Dr. B. Smith-Sørensen (The Norwegian Radium Hospital, Oslo, Norway), whereas the human cDNA clones for *PLK* and *CCNB1* were obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (Berlin, Germany). The human cDNA probe for *CDKN1A* was a gift from Dr. B. Vogelstein (The John Hopkins University School of Medicine, Baltimore, MD). To evaluate the amounts of RNA loaded, the filters were rehybridized to a kinase-labeled oligonucleotide probe complementary to nucleotides 287 to 305 of human 18S rRNA.

Assessment of Clonogenic Regrowth

Clonogenic regrowth efficiency of the MA-11 cell line was determined by plating single cells suspended in media with or without TSA (10–300 nmol/L) for 12 hours, before the media were replaced with fresh media and the cells irradiated. The appropriate plating density was aimed to produce 20 to 40 surviving colonies in each well of six-well culture plates, and the mean plating efficiency of the

control MA-11 cells was not more than ~0.225 in the three independent sets of experiments. After incubation for 2 to 3 weeks, the cells were fixed and stained with 0.1% crystal violet. Colonies of ≥50 cells were counted to determine surviving fraction. At least four parallel samples were scored in the three repetitions done for each treatment condition.

Calculation of Radiation-TSA Interactions

The results from the clonogenic regrowth measurements were analyzed using the combination index method of Chou and Talalay (18). Combination index <0.90 is indicative of synergistic interactions, combination index of 0.90 to 1.0 indicates additive interactions, and combination index >1.1 indicates antagonistic interactions.

Results

HDAC Inhibition—Histone Acetylation and Poly(ADP-Ribose) Polymerase Protein Status

Tumor cell sensitivity to pharmacologic HDAC inhibition may vary along a wide concentration range and should be considered highly cell line specific. Thus, the initial experiment was done to determine the effect of increasing concentrations of TSA (10–300 nmol/L) on the histone acetylation status of the MA-11 cell line. As seen from Fig. 1 (*top*), the level of acetylated histone H4 was substantially induced after 6 to 12 hours of exposure to higher TSA concentrations (100 and 300 nmol/L) before the level again dropped below detection after 24 hours. In contrast, histone H4 acetylation was not seen in cells treated with lower TSA concentrations (10 and 30 nmol/L). The expression pattern of acetylated histone H3 was closely similar (data not shown), which might indicate that MA-11 cell histones are insensitive to TSA below a threshold concentration.

These data suggest that TSA at a concentration of 300 nmol/L might be appropriate for further mechanistic studies. The possibility of MA-11 cell apoptosis at the said TSA concentration (19, 20) was analyzed by means of poly(ADP-ribose) polymerase cleavage as we have recently shown that degradation of this nuclear repair enzyme is a sensitive indicator of apoptotic cell death activated by a *Pseudomonas* exotoxin A–containing immunotoxin in MA-11 cells (21). In contrast to what was detected in the immunotoxin-treated cells, the poly(ADP-ribose) polymerase protein remained intact in MA-11 cells incubated with 300 nmol/L TSA throughout the observation period of 24 hours (Fig. 1, *bottom*), excluding apoptosis as an essential mechanism in TSA-induced MA-11 cell death (see below).

Ionizing Radiation and HDAC Inhibition—Redistribution of Cell Cycle Phases

Pharmacologic inhibition of HDAC activity has been shown to cause cell cycle arrest at the G_2-M boundary in a variety of tumor cell lines (19, 20, 22–24), resembling the G_2 checkpoint response to DNA damage induced by ionizing radiation (10, 12, 25). Hence, MA-11 cells were exposed to a radiation dose of 8.0 Gy or 300 nmol/L TSA, and cell cycle profiles were followed for 24 hours (Fig. 2, *top*).

The irradiated cells displayed an apparent accumulation of G_2-M phase cells throughout the observation period. The fraction of G_2-M phase cells increased from 20% to 25% of the total cell counts after 12 hours to ~40% after 24 hours. A similar redistribution of cell cycle phases was observed in the TSA-treated cells, although a larger cell fraction was in S phase and a significantly lower fraction (~30% of the total cell counts) was arrested in the G_2-M phase after 24 hours, compared with cells exposed to ionizing radiation.

p53 Status

In MA-11 cells, a distinct G_1 phase was detected following both irradiation and TSA treatment (Fig. 2). The persisting G_1 phase cells may reflect an incomplete G_2-M phase arrest, allowing DNA-damaged cells to pass into the

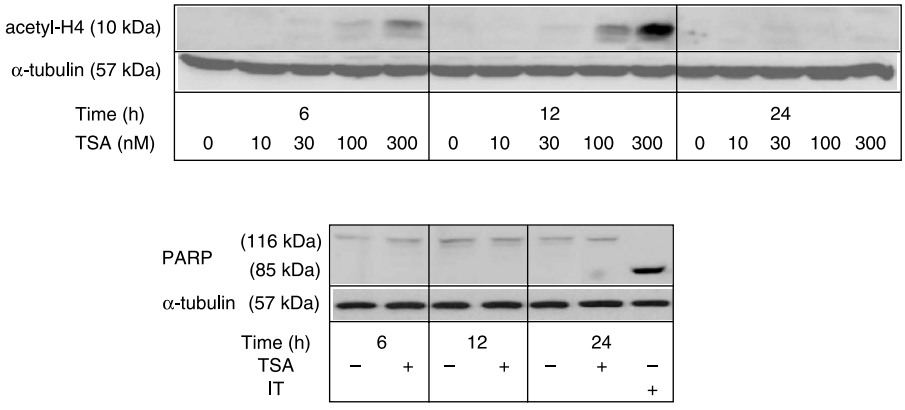

Figure 1. HDAC inhibition by TSA in MA-11 cells—histone acetylation and poly(ADP-ribose) polymerase protein status. The cells were treated with TSA at increasing concentrations (*top*) or at 300 nmol/L (+), or left untreated (–; *bottom*). Protein extracts prepared after 6 to 24 h of incubation were analyzed by Western blot hybridization with an antibody against acetylated histone H4 (*acetyl-H4*) or an anti–poly(ADP-ribose) polymerase (*PARP*) antibody that binds with higher affinity to the poly(ADP-ribose) polymerase cleavage fragment (85 kDa) than to the uncleaved fragment (116 kDa). *Bottom,* included is a protein extract from MA-11 cells treated (+) with 10 ng/mL of a *Pseudomonas* exotoxin A–containing immunotoxin (*IT*), used as positive control for poly(ADP-ribose) polymerase cleavage (21). Expression of α-tubulin was measured as loading control.

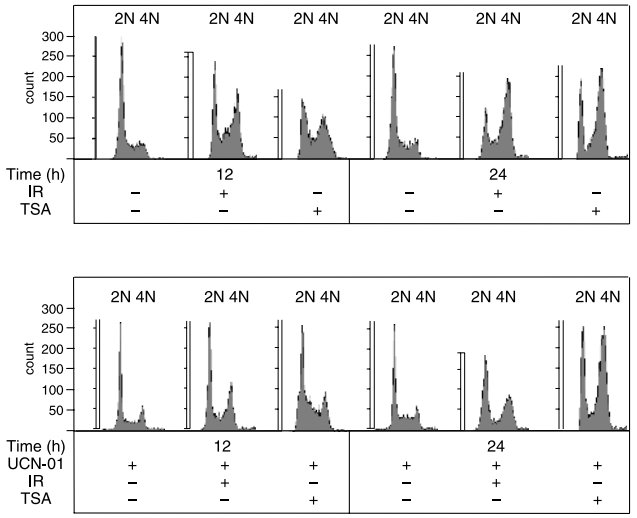

Figure 2. MA-11 cell cycle profiles on exposure to ionizing radiation (*IR*) or the HDAC inhibitor TSA. The cells were treated (+) with ionizing radiation (8.0 Gy) or TSA (300 nmol/L), or left untreated (−), in the absence (*top*) or presence (*bottom*) of the CHK1 inhibitor UCN-01 (100 nmol/L), and further incubated for 12 and 24 h before cellular DNA contents were determined by flow cytometry analysis gated for Hoechst 33258 fluorescence. Scales indicating cell counts (*y axes*) for G_1 and G_2-M phase cells (*2N* and *4N*, respectively) are provided.

G_1 phase of a new cell cycle. Alternatively, the regulatory mechanism of such pattern of cell cycle redistribution may involve a functional G_1 checkpoint. Hence, the cellular p53 status was analyzed and compared with that of the MT-1 cell line, in which cell cycle responses to ionizing radiation have been previously reported (10, 12).

As depicted in Fig. 3 (*top*), constant denaturant gel electrophoresis analysis of DNA from the MA-11 cell line revealed aberrant migration of a PCR fragment representing exon 5 of the *TP53* gene, in accordance with the base substitution of C for a T nucleotide in codon 126, resulting in an amino acid change of Tyr to His, detected by the subsequent sequencing of the PCR fragment. In contrast, we found with DNA from MT-1 cells all exons of the *TP53* gene to be wild-type on constant denaturant gel electrophoresis analysis.

Furthermore, both cell lines showed essentially equal levels of mRNA expression for *TP53* (Fig. 3, *middle*). However, whereas strong p53 protein expression was found in the MA-11 cell line, in accordance with the intense nuclear staining of p53 previously detected by immunocytochemistry (2), p53 expression was almost undetectable in MT-1 cell extracts. A weak band representing the p53 protein was seen after long-term exposure of the immunoblot (Fig. 3, *bottom*).

Primarily, p53 stabilization results from disruption of the interaction between p53 and the MDM2 oncoprotein, which thereby protects p53 from ubiquitin-mediated degradation (26). Whereas expression of two major MDM2 polypeptides was observed in MT-1 cells, MDM2 was almost undetectable in MA-11 cells (Fig. 3, *bottom*).

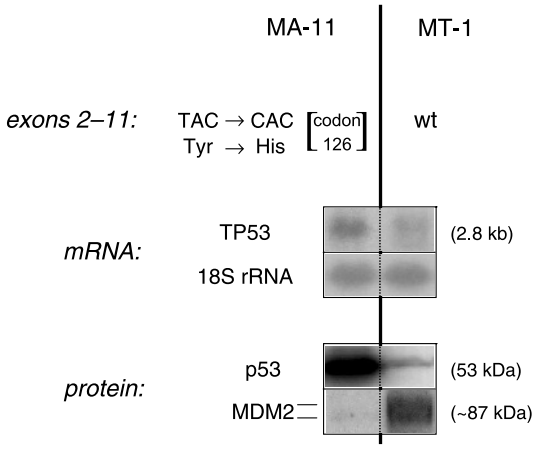

Figure 3. p53 status of MA-11 cells, as compared with that of the MT-1 cell line. *Top,* exons 2 to 11 of the *TP53* gene were individually amplified by PCR from DNA extracted from the cell lines, and further analyzed by the constant denaturant gel electrophoresis method. Any aberrantly migrating PCR fragments were subsequently sequenced. A detected single-base mutation is indicated. *wt,* wild-type. *Middle,TP53* mRNA expression in the cell lines was analyzed by Northern blot hybridization using 18S rRNA as RNA loading control. *Bottom,* protein expression of p53 and MDM2 was determined by Western blot hybridization.

Ionizing Radiation and HDAC Inhibition—Responses of Cell Cycle Regulatory Proteins

The responses of regulatory proteins of the G_1 and G_2 cell cycle phases were followed for 24 hours after exposure of MA-11 cells to ionizing radiation (8.0 Gy) or TSA (300 nmol/L). As seen in Fig. 4, expression of the G_2 phase kinase Plk1 was found to be down-regulated 6 hours after irradiation, with an apparent increase above the control Plk1 level, probably compensatory, after 12 to 24 hours. This has also been observed in irradiated MT-1 cells (12). A transient Plk1 repression was also seen 6 to 12 hours after start of the TSA treatment but almost recovered after 24 hours. From below detection, the level of the G_1 phase

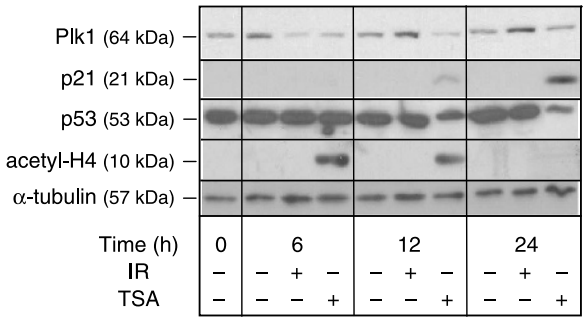

Figure 4. Checkpoint regulatory proteins in MA-11 cells after exposure to ionizing radiation (*IR*) or the HDAC inhibitor TSA. The cells were treated (+) with ionizing radiation (8.0 Gy) or TSA (300 nmol/L), or left untreated (−), and protein expression levels of Plk1, p21, p53, and acetylated histone H4 (*acetyl-H4*) after 0 to 24 hours were determined by Western blot analysis of cell extracts. Expression of α-tubulin was measured as loading control.

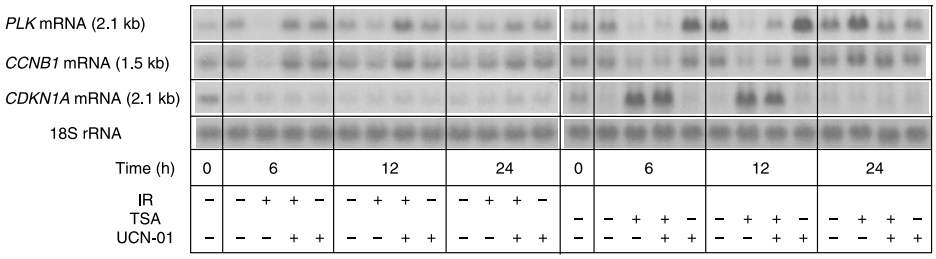

Figure 5. Cell cycle regulatory factors and CHK1 signaling after exposure of MA-11 cells to ionizing radiation (*IR*) or the HDAC inhibitor TSA. The cells were treated (+) with ionizing radiation (8.0 Gy) or TSA (300 nmol/L), or left untreated (−), in the absence (−) or presence (+) of the CHK1 inhibitor UCN-01 (100 nmol/L). Expression levels of mRNAs for *PLK*, *CCNB1*, and *CDKN1A* after 0 to 24 h were analyzed by Northern blot hybridization. 18S rRNA was used as RNA loading control.

inhibitor p21 was induced 12 hours after addition of TSA and further observed as clearly accumulating after 24 hours. Coincident with this, the high level of p53 was substantially repressed. In contrast to the TSA-dependent effects on p21 and p53, the expression levels of these proteins were not altered by radiation.

The regulatory responses of cell cycle proteins seemed to reflect changes in mRNA levels after exposure of MA-11 cells to ionizing radiation or TSA (Fig. 5). As previously observed in other breast cancer cell lines (10, 12, 25), the level of *PLK* mRNA (encoding Plk1) was barely detectable 6 hours after irradiation but almost recovered after 12 hours, and the response of *CCNB1* mRNA (encoding the G_2 phase–specific cyclin B1) was essentially identical. The transient down-regulation of these mRNAs by TSA was observed for a longer period (6–12 hours) before their expression again was up-regulated 24 hours after start of the TSA treatment. The mRNA expression of *CDKN1A* (encoding p21) was not altered after irradiation, which is highly indicative of a defective G_1 checkpoint (7). In the presence of TSA, however, expression of *CDKN1A* mRNA inversely reflected the regulatory effects on *PLK* and *CCNB1* mRNAs.

Ionizing Radiation and HDAC Inhibition—Regulatory Role of CHK1

We have recently shown that the cell cycle phenotype responses following radiation-induced DNA damage require intact G_2 checkpoint signaling through the downstream checkpoint kinase CHK1 (10, 12). In accordance with this, treatment with the CHK1 inhibitor UCN-01 (100 nmol/L) also seemed to counteract the G_2-M phase arrest observed 12 to 24 hours after exposure of MA-11 cells to a radiation dose of 8.0 Gy, but not on incubation with TSA (300 nmol/L) for 24 hours, as seen by comparing the histograms in Fig. 2 (*top* and *bottom*). Furthermore, whereas the suppression of *PLK* and *CCNB1* mRNA expression following irradiation of MA-11 cells was entirely abolished by UCN-01, the regulatory effects of TSA on these mRNAs, and on *CDKN1A* mRNA, were not (Fig. 5). Hence, our data strongly indicate that the effector mechanisms of the G_2 phase responses to ionizing radiation and HDAC inhibition are mediated via distinct regulatory pathways.

Ionizing Radiation and HDAC Inhibition—Clonogenic Regrowth

Finally, the MA-11 cell line was exposed to increasing doses of ionizing radiation to determine clonogenic survival (Fig. 6). The cell line showed nearly exponential loss of colony formation efficiency, with a surviving fraction of ~0.01 with the highest radiation dose applied (10 Gy).

Because the regulatory pathway recruited by HDAC inhibition seemed to be distinctly different from that following irradiation, the possible radiosensitizing effect of TSA, essentially by amplifying the cytotoxic effect of ionizing radiation on clonogenic regrowth, was measured. Based on the histone acetylation data (Fig. 1, *top*), we chose

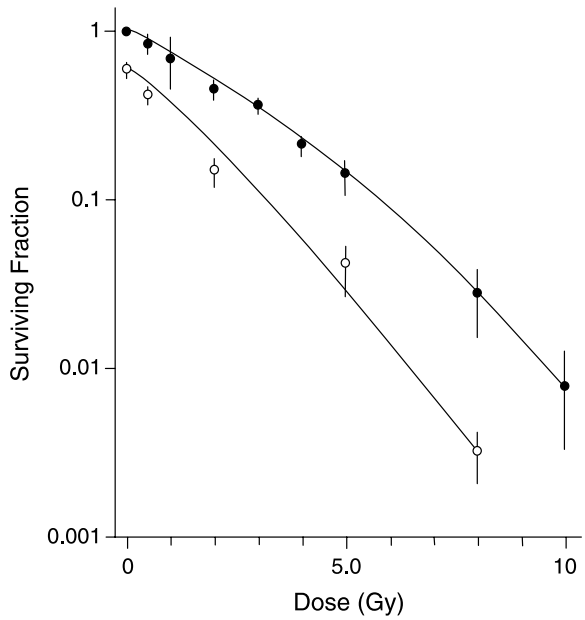

Figure 6. Inhibition of HDAC activity modulates the outcome of MA-11 clonogenic regrowth on exposure to ionizing radiation. MA-11 cells were exposed to increasing doses of ionizing radiation without (●) or following (○) pretreatment for 12 h with TSA (300 nmol/L) to determine relative colony formation compared with the unirradiated control. *Points,* mean (n = 3); *bars,* SE.

to analyze MA-11 cells treated with TSA (10–300 nmol/L) for 12 hours before the HDAC inhibitor was removed and the cells irradiated. This treatment strategy was supported by the observations that irradiation followed by the immediate TSA treatment for 12 hours did not reduce MA-11 cell clonogenicity compared with irradiation alone, and that TSA incubation for 24 hours or more before or after radiation exposure induced complete cytotoxicity in this cell line (results not shown).

The effects of TSA pretreatment in combination with radiation at different doses were measured (Fig. 6) and analyzed by means of the combination index method (Table 1). In most of the doses analyzed (0.50–5.0 Gy), the ability of clonogenic regrowth of irradiated MA-11 cells was reduced by a factor of ≤3 after pretreatment with 300 nmol/L TSA. The cytotoxic effect of 8.0 Gy of ionizing radiation on clonogenicity (surviving fraction of ~0.04), however, was ~10-fold amplified by TSA (Fig. 6). Moreover, as depicted in Table 1, synergistic effects (combination index <0.90) on MA-11 colony formation were found for most combinations of TSA and ionizing radiation tested, but, interestingly, TSA in the lower concentration range (10 and 30 nmol/L) seemed to antagonize the cytotoxic effect of the 5.0 Gy radiation dose on MA-11 cells.

Discussion

In this report, we have compared cell cycle responses of the human breast carcinoma MA-11 cell line to ionizing radiation and HDAC inhibition. Whereas accumulation of G_2-M phase cells, as well as the preceding repression of the genes encoding the G_2 phase regulators Plk1 and cyclin B1, after irradiation required intact G_2 checkpoint signaling through the checkpoint kinase CHK1, the similar phenotypic changes observed with HDAC inhibition did not. MA-11 cells did not show radiation-dependent induction of the G_1 phase inhibitor p21, indicative of a defective G_1 checkpoint and possibly consistent with the base substitution detected in the tumor suppressor *TP53* gene. Induction of p21, however, was observed with HDAC inhibition. Following pretreatment with the HDAC inhibitor, the efficiency of clonogenic regrowth after irradiation was reduced, which is in accordance with the concept of increased probability of mitotic cell death when the chromatin structure is disrupted.

Table 1. Combination index values for ionizing radiation plus TSA

Ionizing radiation (Gy)	TSA (nmol/L)	Combination index
2.0	10	0.67
2.0	30	0.67
2.0	100	0.51
2.0	300	0.51
5.0	10	1.20
5.0	30	1.10
5.0	100	0.88
5.0	300	0.73
8.0	10	0.78
8.0	30	0.77
8.0	100	0.43
8.0	300	0.29

Recent reports have shown that HDAC inhibitors possess antiproliferative effects in a variety of tumor cell lines (19, 20, 24). Moreover, the potential of HDAC inhibitors to sensitize tumor cells to the DNA-damaging cytotoxicity of chemotherapeutics and ionizing radiation has lately been observed (27–32). Interestingly, in animal models, treatment with HDAC inhibitors substantially suppressed the cutaneous side effects of radiation therapy (33), suggesting that the contemporary approach of molecular targeted therapy may be used to increase the therapeutic ratio between the tumor and surrounding normal tissues in radiation therapy.

The clonogenic survival data clearly indicated that TSA in a wide concentration range may sensitize MA-11 cells to the cytotoxic effect of ionizing radiation, although a threshold concentration of the HDAC inhibitor seemed to be necessary to obtain histone acetylation, as measured by the expression of acetylated histones H4 and H3. It has been shown that TSA also acts via mechanisms involving hyperacetylation of nonhistone proteins (22). Although TSA seemed to cause acetylation of histones and nonhistone proteins within the same concentration range, accumulation of acetylated nonhistone proteins occurred more rapidly. This difference in TSA responses might be of consequence for cellular toxicity (22) and perhaps account for the apparent antagonism observed with low concentrations of TSA in MA-11 cells exposed to 5.0 Gy of ionizing radiation as well.

Pharmacologic inhibition of HDAC activity has previously been shown to cause redistribution of cell cycle profiles resembling the G_2 checkpoint response to DNA damage induced by ionizing radiation (19, 20, 22–24). *PLK* and *CCNB1* are among the several genes encoding mitotic regulators, of which the mRNA expression levels are down-regulated following activation of the G_2 checkpoint (34). The present report is the first on TSA-directed down-regulation of *PLK* mRNA. Whether this is regulated at the level of transcription, similar to what has been shown for the repressed *CCNB1* promoter activity with HDAC inhibition (23, 35, 36), remains to be determined.

The promoter of the human *PLK* contains a characteristic repressor element in the region of the transcription start site, which mediates the cell cycle phase–specific regulation of the gene expression (37). This repressor element is involved in the inhibition of *PLK* transcription after activation of p21 (38), which can function as a highly specific transcriptional regulator of numerous genes involved in cell cycle progression and DNA repair (39). However, the observation that *PLK* mRNA repression by TSA clearly preceded the induction of p21 argues against a p21-directed pathway as the principal effector mechanism.

Treatment with HDAC inhibitors induces p21 expression through a transcriptional mechanism, possibly mediated by the ATM kinase signaling pathway (40) and associated changes in the acetylation status of the *CDKN1A* promoter (23, 41). Accordingly, we found that both *CDKN1A* mRNA and the p21 protein were induced from very low baseline levels after addition of TSA. However, the effector mechanism of this p21 accumulation, supposed to be initiated by ATM, did not seem to involve the downstream checkpoint kinase CHK1, which suggests diversity in the regulatory pathways governed by ATM in DNA damage checkpoint control. Similarly, our data on repression of Plk1 and cyclin B1 followed by G_2-M phase arrest after exposure to ionizing radiation and TSA strongly indicate that these G_2 phase responses, although phenotypically similar, are mediated via distinct regulatory pathways.

In MA-11 cells, a distinct G_1 phase was detected following both irradiation and TSA treatment. The regulatory mechanism of such pattern of cell cycle redistribution might involve a functional G_1 checkpoint, in the event of which a radiation-induced up-regulation of *CDKN1A* mRNA is observed (7). However, the complete lack of such a response supports the assumption that the persisting G_1 phase reflects an incomplete G_2 phase arrest.

Consistent with a defective G_1 checkpoint, a base substitution in codon 126 of the *TP53* gene and a correspondingly high p53 protein level were detected in the MA-11 cell line. Primarily, p53 stabilization results from disruption of the interaction between p53 and the MDM2 oncoprotein, which thereby protects p53 from ubiquitin-mediated degradation (26). The expression of MDM2 was almost undetectable in MA-11 cells. This is in accordance with the concept that tumor cells with high intrinsic levels of mutant inactive p53 are unable to induce expression of the MDM2 protein, which would normally provide a feedback mechanism of p53 destabilization in the absence of DNA-damaging events (42).

Based on the frequency of recorded *TP53* mutations,[3] base substitutions in codon 126 are rare, but a few examples of missense mutations are reported [e.g., in head-and-neck cancer (43, 44) and metastatic lesions from prostate cancer (45)].

Basically, this report describes proof-of-principle experiments on how to use HDAC inhibitors to decrease the probability of clonogenic regrowth of tumor cells exposed to ionizing radiation. Although appealing as a concept, caution must be shown with interpretation and, indeed, possible therapeutic utilization. There are currently several HDAC inhibitors in early-phase clinical trials (14). It is reasonable to believe that therapeutic indications for these agents primarily will be in combination with conventional cytotoxic therapies.

[3] http://p53.genome.ad.jp

Acknowledgments

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