Evasion of Early Cellular Response Mechanisms following Low Level Radiation-induced DNA Damage*

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DNA damage that is not repaired with high fidelity can lead to chromosomal aberrations or mitotic cell death. To date, it is unclear what factors control the ultimate fate of a cell receiving low levels of DNA damage (i.e. survival at the risk of increased mutation or cell death). We investigated whether DNA damage could be introduced into human cells at a level and frequency that could evade detection by cellular sensors of DNA damage. To achieve this, we exposed cells to equivalent doses of ionizing radiation delivered at either a high dose rate (HDR) or a continuous low dose rate (LDR). We observed reduced activation of the DNA damage sensor ataxia-telangiectasia mutated (ATM) and its downstream target histone H2A variant (H2AX) following LDR compared with HDR exposures in both cancerous and normal human cells. This lack of DNA damage signaling was associated with increased amounts of cell killing following LDR exposures. Increased killing by LDR radiation has been previously termed the “inverse dose rate effect,” an effect for which no clear molecular processes have been described. These LDR effects could be abrogated by the preactivation of ATM or simulated in HDR-treated cells by inhibiting ATM function. These data are the first to demonstrate that DNA damage introduced at a reduced rate does not activate the DNA damage sensor ATM and that failure to activate ATM-associated repair pathways contributes to the increased lethality of continuous LDR radiation exposures. This inactivation may reflect one strategy by which cells avoid accumulating mutations as a result of error-prone DNA repair and may have a broad range of implications for carcinogenesis and, potentially, the clinical treatment of solid tumors.

Ionizing radiation (IR) causes numerous types of DNA damage, which occur at different frequencies within the cell (1). However, it is generally accepted that DNA double strand breaks (DSBs) are the most important type of damage with respect to the latent effects of radiation exposure such as chromosomal aberrations, tumorigenesis, and cell death (2–4). For a cell to survive the potentially lethal damage conferred by radiation exposure, DNA DSBs must be rapidly detected, and DNA repair mechanisms must be initiated. However, in humans, the primary DSB repair pathway is somewhat error-prone, and therefore, the fidelity of the genome is not always maintained (2–5). This may be particularly important following the production of low levels of DNA damage in which cellular detection mechanisms may not be fully elicited (5, 6). Evidence exists (7) that increased cell death in the absence of repair following low level DNA damage may offer a way by which the cell prevents potentially promutagenic lesions from being passed on to progeny.

It has been shown recently (8–11) that within minutes of incurring small amounts of DNA damage, the critical damage sensor molecule ATM is activated by an autophosphorylation event at Ser-1981, which might require the NBS1 protein. Once activated, ATM is responsible for initiating several signaling cascades, which are essential to halt cell cycle progression that allows the DNA damage to be repaired (see Ref. 12 and references therein). One of the earliest detectable downstream targets of ATM is the histone H2A variant, H2AX, which is phosphorylated by ATM at Ser-139 (13). It appears that phosphorylated H2AX (termed γH2AX) encompasses a region of several thousand base pairs around the damage sites, forming foci within the nucleus that act as a molecular beacon signaling for the recruitment of DNA repair factors (14–16). Indeed, mice and cells lacking H2AX show chromosomal instability and defective DNA repair (15, 17).

Recently, γH2AX has been shown (18–20) to be a reliable marker of the number of DNA DSBs produced in a cell following exposure to DNA damaging agents such as IR. Furthermore, Rothkamm and Löbrich (20) recently demonstrated that this rapid activation of H2AX occurs following even very low doses (1 mGy) of IR given at high dose rates. They also showed that the surviving fraction of cell cultures exposed to very low doses of IR was lower than would be predicted from dose–response curves, suggesting that a threshold of damage is reached before cellular detection mechanisms are efficiently activated. The existence of such a DNA damage threshold is supported by the phenomenon known as low dose hyper-radiosensitivity, which is the default response of the majority of cell cultures exposed to either low doses of IR (7) or to radiation delivered at a low dose rate (i.e. a reduced amount of radiation dose/unit of time but amounting to the same total dose of radiation) (21).

Given these previous studies, we were interested in investigating whether low levels of DNA damage were capable of evading these early cellular DNA damage detection mecha-
nisms, which would predictably lead to enhanced cell death. To achieve continuous low levels of DNA damage, we irradiated cells at low dose rates (LDR) of IR, which produces ~4–5 DNA DSBs/h. These dose rates are ~450 times less than the high dose rates (HDRs) typically employed in conducting in vitro and in vivo experimental research (which produces ~1800 DNA DSBs/h) and are used in the HDR experiments reported here. Although the same total dose of IR is delivered to the cell in both HDR and LDR exposures, LDR radiation exposure is generally accepted as a potentially less damaging modality given that the cells are exposed to a lower radiation dose/unit of time (6, 22). In all four of the different human cancer cell lines we used, we observed greater amounts of cell killing (reduced clonogenic capacity) following 2 Gy delivered at a LDR compared with cells given the same total dose at a HDR. Importantly, we demonstrate that the increased cell killing of LDR-treated cells is a consequence of inefficient activation of the DNA damage sensor ATM and its downstream target H2AX, which acts as a cellular signal of DNA damage. Similar results were also seen in normal human primary fibroblasts, and thus inefficient activation may represent a general method by which cellular DNA damage mechanisms can be evaded and potential mutations avoided. These findings give insight into how low levels of DNA damage are detected within the cell and may aid in further understanding such early cellular DNA damage response mechanisms. These findings also have broad implications for carcinogenesis mechanisms and, potentially, for the clinical treatment of solid tumors.

MATERIALS AND METHODS

Cell Lines and Culture Techniques—All cell lines were obtained from the American Type Culture Collection and maintained as adherent monolayer cultures in appropriate media as outlined in their respective product data sheets. All cultures were grown at 37 °C in a humidified atmosphere of 5% carbon dioxide, fed every 5 days (every 3 days for fibroblasts) with complete medium, and subcultured when confluence was reached. Transfection with siRNA plasmids and subsequent FACS procedures were carried out as described previously (23). For all chloroquine experiments, subconfluent cell cultures were pretreated 4 h prior to radiation exposure with 25 μg/ml chloroquine, which was removed prior to trypsinization and replating into 10-cm culture dishes (clonogenic survival assays) or fixing (H2AX assays).

Clonogenic Survival Assays—For HDR exposures, cell monolayers were trypsinized, counted, and diluted to the appropriate cell density and loaded into 100-mm culture dishes to yield at least 50 colonies/dish following irradiation. Cells were then irradiated to ~4500 cGy/h to the desired dose using a Gamacell 40 137Cs irradiator (Atomic Energy of Canada, Ltd., Ottawa). At 7–14 days after irradiation, colonies consisting of at least 50 cells were stained with 50% crystal violet (Sigma) and counted. Cell survival was plotted as a function of dose and fitted using the linear quadratic model, S = e(−αD−βD2), where e is exponent, S is the cell survival, D is the dose of radiation, and α and β are constants. For LDR exposures, subconfluent cell monolayers were irradiated using a custom-built low dose rate irradiator (24) at a LDR of 9.4 Gy/h or a very low dose rate (VLDLDR) of 2 cGy/h to the desired final dose. For low level radiation exposures, sealed flasks were maintained at 37 °C in the low dose rate irradiator for the desired time. As controls, unirradiated flasks were also sealed and incubated at 37 °C for an equivalent amount of time. Following IR exposures, cells were trypsinized, counted, and diluted to the appropriate cell density into 10-cm culture dishes to give at least 50 colonies/dish following 7–14 days of growth after plating. Surviving fractions were calculated as for HDR experiments and then corrected for cell loss during the protracted radiation exposures as described previously (21). All statistical analyses (two-tailed independent t-tests) were performed using Microsoft Excel.

FACS Analysis of γH2AX Activation—Subconfluent cell monolayers were irradiated as described above and fixed in 70% cold ethanol for 45 min following the completion of irradiation. Cells were then stained for activated γH2AX with an antibody specific for phosphorylated Ser-139 (Upstate Biotechnology, Waltham, MA) using the protocol described by MacPhail et al. (19). Stained cells were analyzed on a LSR flow cytometer (BD Biosciences), and the relative amount of fluorescence in each cell population was determined using the BD Biosciences CellQuest program.

Immuno blot—Whole cell extracts were collected at 15 min postirradiation exposure and separated on 4–15% acrylamide gels (Bio-Rad) using standard SDS-PAGE techniques. Antibodies for ATM, phosphospecific ATM (Ser-1981), phospho-specific NBS1 (Ser-345), NBS1, and β-actin were obtained from Dr. Michael Kastan (St. Jude Children’s Research Hospital, Memphis, TN), Upstate Biotechnology, Novus Biologicals (Littleton, CO), and Sigma, respectively. A total of 10–20 μg of protein extracted from each transfected cell population was loaded onto each gel, electrophoresed at 100 V for 3 h at 4 °C, and then transferred overnight at 50 mA onto polyvinylidene difluoride membranes (Bio-Rad) at 4 °C. Membranes were probed with both primary and secondary antibodies at optimized concentrations, and protein expression was visualized using an ECL kit (Amersham Biosciences). Membranes were probed for β-actin to normalize for loading errors. Protein expression was quantified using a Versa-Doc gel documentation system (Bio-Rad). For immunoprecipitation Western blots, lysed whole cell extracts were mixed with 0.5 μg of ATM or NBS1-P antibody (courtesy of Dr. Michael Kastan and Upstate Biotechnology, respectively) for 1 h at 4 °C prior to binding to protein G-linked agarose beads (1 h incubation at 4 °C). Samples were then lysed by boiling for 5 min in the presence of 2% SDS loading buffer. Approximately half of each sample was loaded onto a 4–15% acrylamide gel and probed for phospho-specific ATM as described above. Membranes were then stripped and reprobed for ATM or NBS1 as described above. The DU145 cell line used for these experiments had been shown previously (9–11) to express all three components of the MRE11-RAD50-NBS1 complex, which has been shown recently to play a role in the activation of ATM following DNA damage.

RESULTS

Low Level Radiation-induced DNA Damage Results in Increased Cell Death—To ascertain whether greater amounts of cell killing could be achieved by low level radiation damage, we exposed a panel of human cancer cell lines to 2 Gy of IR delivered at either a HDR of ~4500 cGy/h, which is typically used in both clinical practice and experimental research, or a LDR of ~9.4 cGy/h and assessed clonogenic survival. We observed a general trend for greater cell killing following radiation exposures delivered at the LDR compared with the HDR in all cell lines tested (Fig. 1). This was statistically significant (p < 0.05) in two (RKO and DU145) of the four cell lines tested. The observation of enhanced cell killing following LDR radiation exposures compared with the more common HDR is well known but poorly understood phenomenon in the radiation biology field and is termed the inverse dose rate effect (see Ref. 25).

Early DNA Damage Responses Are Abrogated following Low Level Radiation Exposure—We were interested in determining whether attenuation in the recognition of DNA damage could explain the inverse dose rate phenomenon. We hypothesized that the recently identified rapid activation of ATM and its downstream target H2AX following IR exposure might be reduced or abrogated in cells receiving LDR radiation compared with those receiving HDR exposures. Using the two cancer cell lines that exhibited a statistically significant reduced survival following LDR exposures (Fig. 1), we showed that the activation of ATM (phosphorylation of Ser-1981) was reduced by approximately 40–50% following LDR radiation exposure compared with cells treated with equivalent doses of radiation delivered at the HDR (Fig. 2A). LDR exposure also led to a reduction (~25%) in levels of phosphorylated NBS1 (Ser-343) and concurrently in a reduced (~25%) association of ATM with phosphorylated NBS1 (Fig. 2B). Reduced activation of ATM was also seen in the normal human primary fibroblasts (Fig. 2C), which were used by Bakkenist and Kastan (8) to identify the rapid phosphorylation of ATM at Ser-1981 following IR exposure. To determine whether LDR exposure would result in the activation of processes that prevent ATM phos-
phorylation, we exposed DU145 cells to a 2-Gy LDR followed immediately by a 6-Gy HDR. Cells that were preirradiated with LDR IR were capable of further phosphorylation of ATM at Ser-1981 on exposure to HDR radiation (Fig. 2). This suggests that the low levels of activated ATM seen following LDR radiation exposure are not the consequence of a mutation to the Ser-1981 residue, the presence of a dysfunctional ATM protein, or the activation of inhibitory processes.

The histone variant H2AX is considered a marker of DNA damage detected as the result of insults such as ionizing radiation (18, 19, 26–28). It is one of the first downstream targets of ATM, becoming phosphorylated on Ser-139 within 1–3 min following DNA damage (13, 29), thereby promoting DNA repair processes. We were interested in determining whether the low levels of activated ATM seen following LDR radiation also resulted in a reduction in the phosphorylation and activation of H2AX. We performed FACS analyses to quantify γ-H2AX levels at appropriate times after exposing RKO and DU145 cells to 2 Gy of IR, delivered at either the HDR or the LDR. As with the activation of ATM, γ-H2AX levels were statistically lower in cell cultures exposed at the LDR compared with the HDR-treated cells (Fig. 3).

Low Level DNA Damage Results in Reduced Signaling across a Range of IR Doses and Is Ubiquitous throughout the Cell Cycle—Although we observed that the surviving fraction of cells following a 2-Gy dose of IR was lower in cells exposed to LDR compared with HDR exposures (Fig. 1), we were interested to see the effects of LDR exposures over a range of IR doses. Exposure of DU145 cells to increasing doses of IR revealed that clonogenic survival was significantly reduced in cell cultures treated with LDR IR compared with HDR IR at doses between 0 and 6 Gy and that this difference increased dramatically as the total dose increased (Fig. 4). This increased cell killing is thought to be caused by a decrease in the clonogenic capacity of the cells following LDR IR exposures because of the inefficient activation of DNA repair processes. Another possible cause for increased cell killing could be an accumulation of senescent cells following IR exposure before replating for the clonogenic assays. However, analysis of the senescence marker senescence-associated β-galactosidase following HDR- and
LDR-treated cells showed no differences in expression levels compared with unirradiated controls or between HDR- and LDR-treated cells (data not shown).

To assess the relationship between the detection of DNA double strand breaks and radiation dose rate, we carried out FACS-based analyses to assess levels of γ-H2AX following HDR and LDR IR exposures across a range of doses. Similar to the data shown in Fig. 3, we observed decreased levels of γ-H2AX in cell cultures irradiated between 0 and 6 Gy with a LDR compared with a HDR. As with clonogenic survival (Fig. 4A), this difference became more apparent as the total dose increased (Fig. 4B). Further analysis of γ-H2AX activation revealed that γ-H2AX levels following a LDR was lower in all phases of the cell cycle compared with cells exposed to equivalent radiation doses delivered at the HDR and thus was not cell cycle-dependent (Fig. 4C). These data are consistent with an overall inefficient detection of DNA DSBs caused by the reduced activation of early cellular DNA damage response mechanisms following LDR, leading ultimately to greater amounts of cell killing per radiation dose compared with HDR-treated cells (Fig. 4D). This increased cell killing is likely caused by a failure to sufficiently activate DNA damage checkpoints and DNA repair mechanisms before cell division takes place.

The Role of ATM in Early Cellular Responses to Low Level DNA Damage and Modulation of ATM Activity in LDR- and HDR-treated Cells—To further investigate the role of ATM in cellular responses to low level DNA damage following LDR radiation exposures, we pretreated DU145 cells with chloroquine 4 h prior to the initiation of radiation exposures. Chloroquine has been shown previously (8) to activate ATM without the production of DNA DSBs and thus does not affect γ-H2AX levels (data not shown). Cells pretreated with chloroquine prior to LDR radiation exposure have demonstrated both clonogenic survival and γ-H2AX levels on a par with HDR-treated cells (Fig. 5A and B), that is at levels representing “normal” cellular responses to a 2-Gy IR exposure.

In accordance with these data, we noticed that the increased amount of cell killing of DU145 cells seen in LDR-treated cultures mimicked that observed in HDR-treated DU145 cells in which ATM had been knocked down by ~80–90% following siRNA-mediated inhibition (23). We were therefore interested to see whether we could reproduce these data and whether siRNA-mediated inhibition of ATM prior to HDR radiation exposure would yield reduced amounts of γ-H2AX, as observed in LDR-treated cells. To achieve this, we assessed clonogenic survival and γ-H2AX levels following 2-Gy HDR and LDR exposures in DU145 cells, which had been transfected prior to the start of the radiation exposures with an anti-ATM siRNA-encoding plasmid as described previously (23). As predicted, cells transfected with the anti-ATM siRNA plasmid showed statistically significant reductions in clonogenic survival and γ-H2AX activation following a 2-Gy HDR exposure compared with untransfected controls (Fig. 6, A and B). Both clonogenic survival and levels of γ-H2AX in HDR-irradiated siRNA-treated cells were similar to those seen in untransfected LDR-treated cells (Fig. 6, A and B), highlighting the importance of ATM in early cellular responses to radiation-mediated damage. The amounts of γ-H2AX observed in ATM siRNA-treated cells were similar to the residual levels reported (13, 30) for irradiated mutant ATM cells and were likely caused by small amounts (−10%) of ATM still present in the cell following siRNA treatment (23).

Finally, we were interested in determining whether reducing the dose rate further would result in lower levels of activated ATM and H2AX, culminating in greater amounts of cell killing compared with that seen at 9.4 cGy/h. To study this, we irradiated DU145 cells at a VLD of 2 cGy/h, producing −1 DNA DSB/h, and observed an increased amount of cell killing in these cells compared with HDR-treated cells (Fig. 7A). Importantly, the surviving fraction of VLD-treated cells was lower than that seen for LDR-treated cells (p = 0.03). Western blot analyses showed drastically reduced activated ATM levels following a 2-Gy VLD radiation exposure (Fig. 7B). Finally, we carried out FACS analyses for activated H2AX in VLD-treated cells. Although γ-H2AX levels were much lower in VLD-treated compared with HDR-treated cells, we did not see any significant reduction in γ-H2AX levels in VLD-treated compared with LDR-treated cells (Figs. 3 and 7C). This observation may reflect a limitation of the FACS assay used, or it may be that H2AX can be activated in ATM-independent mechanisms, for example, by either DNA-protein kinase catalytic subunit (DNA-PKcs) (31, 32) or NBS1 (9–11). We are currently investigating the roles of these proteins in cellular responses to LDR radiation treatments.

DISCUSSION

DNA damage and induced repair are generally accepted as critical components of cell survival following exposure to IR. These same processes have been implicated in the onset of carcinogenesis following exposure to radiation and other DNA damaging agents, as well as in endogenous DNA breaks (2–5). Recent work (8–11, 13, 14, 19, 27, 31) has highlighted both the importance of early cellular DNA damage detection mechanisms in eliciting the necessary responses that lead to the eventual repair of DNA DSBs and the intrinsic sensitivity of these activation events to low levels of DNA damage (8, 10, 20). Although these
early DNA damage response mechanisms are extremely sensitive, low levels of DNA damage (DSBs) can fail to sufficiently activate appropriate repair mechanisms (20). We therefore investigated whether low levels of DNA damage produced by ionizing radiation given at a reduced rate were capable of evading early cellular DNA damage detection mechanisms.

To achieve continuous low levels of DNA damage, we exposed cells to protracted doses of LDR radiation. The rate used was ~450 times less than the HDRs normally used in clinical practice and in routine in vitro and in vivo experimental research. Although the same total dose of IR is delivered to the cell, LDR radiation exposure is generally accepted as less damaging (in terms of the amount of DNA damage produced; ~4–5 DNA DSBs/h for LDR and ~1800/h for HDR), given that ongoing DNA repair processes may counteract the damage produced during the time taken to deliver the radiation (22).

Using four human cancer cell lines of both colorectal and prostatic origin, we show that greater amounts of cell killing...
can be achieved following LDR exposures compared with HDR exposures (Figs. 1 and 4A). This inverse dose rate effect on cell survival has been known to exist for many cell types, although a complete explanation for its cause has been lacking (21, 24, 33–35). A similar inverse dose rate effect is also known to exist in the production of genomic mutations following radiation exposure (6), which may be a consequence of the ineffective activation of repair mechanisms, as shown by the data presented here. Thus, increased cell death following low level DNA damage as a consequence of reduced repair or a reduction in the initiation of repair signals may represent a default mechanism by which the cell minimizes the likelihood of passing on promutagenic lesions to its progeny. We are currently testing this hypothesis.

The most accepted hypothesis for this inverse dose rate survival phenomenon is that protracted radiation exposure causes cells to accumulate in the $G_2$ phase of the cell cycle, which is the phase most sensitive to ionizing radiation, probably as a consequence of a reduction in the amount of time needed to repair the damage prior to cell division (22, 24, 35). However, more recent studies (21, 36, 37) have observed no direct correlation between radiation sensitivity to such low level protracted exposures (6) and cell cycle distributions. Interestingly, we did not observe a $G_2$ cell cycle arrest in cells following either LDR or VLDR radiation exposures (data not shown), suggesting insufficient DNA damage signaling events in low dose rate-treated cells. We were therefore interested in determining whether early DNA damage response mechanisms, that is, activation of ATM and H2AX, could explain the increased cell killing we observed following LDR IR exposures.

We report here that levels of activated ATM (measured by the phosphorylation of Ser-1981 (8)) were dramatically reduced in human cancer cell lines following LDR-treated cells (Fig. 2A). These cells also showed reduced levels of phosphorylated NBS1 (Ser-343, a target of ATM kinase activity) as well as a reduction in the amount of ATM associated with phosphorylated NBS1 (Fig. 2B), an association that has been shown recently (9–11) to augment the activation of ATM following DNA damage. Reduced activation of ATM was also seen in LDR-treated normal human fibroblasts (Fig. 2C). These fibroblasts were used previously (8) to elucidate the rapid activation of ATM following low amounts of DNA damage produced from HDR IR exposure. It is important to note that this reduced activation of ATM represents an effect that is not caused simply by the longer time it takes to deliver 2 Gy at the LDR compared with the HDR exposures (21 h compared with 2.5 min, respectively), during which time the activation of ATM might have since subsided. It has been recently shown (8) that levels of activated ATM are at a maximum following a total dose of 0.4 Gy and persist for at least 24 h. Although it takes approximately 21 h to deliver 2 Gy at the LDR of 9.4 cGy/h, the threshold of activation (~0.4 Gy) should be reached every 4 h at this dose rate. Furthermore, the reduced levels of activated ATM in LDR-treated cells cannot be explained by the presence of a dysfunctional protein or the activation of inhibitory processes following the radiation exposure because cells exposed to a 2-Gy LDR followed immediately by a 6-Gy HDR showed an
efficient ATM response (Fig. 2D). We further showed that the activation of one of the immediate downstream targets of ATM, H2AX (a marker of cellular detection of DNA DSBs), is also abrogated following LDR IR exposures compared with HDR exposures (Figs. 3 and 4).

Although reduced H2AX activation was not shown to be cell cycle phase-dependent (Fig. 4C), intriguingly, 9.4 cGy/h LDR-treated cells failed to elicit a G2 arrest (data not shown), a response that is normally seen in cells treated with higher LDR radiation exposures (e.g. 25 cGy/h (37)). Our data are thus intriguing given the recent findings that H2AX is involved in the G2/M cell cycle checkpoint following exposure to low but not high levels of DNA damage (30) and by the identification of a novel G2/M checkpoint that is dependent on ATM (38). Interestingly, induction of this novel checkpoint is dose-independent once a total dose of 0.4 Gy (the dose taken to fully activate ATM (8)) has been reached and may be an important factor in determining cellular sensitivity to low levels of DNA damage caused by IR (39, 40). Thus, the lack of ATM activation and lower γ-H2AX in LDR-treated cells resulted ultimately in greater cell killing, caused possibly by the ineffective activation of cell cycle checkpoints, compared with equivalent doses of DNA damage inflicted at higher dose rates (Fig. 4D), such as those commonly used in both experimental and clinically based studies.

There may be several alternative explanations for the reduced H2AX activation seen in LDR-treated cells. The most likely is that the levels of activated H2AX have fallen back toward basal levels during the time in which the radiation was delivered. This temporal activation of H2AX may be important in the data presented here, given that the half-life of H2AX in DU145 cells is ~4.5 h following HDR exposures (19). However, we found that the levels of γ-H2AX in cells treated with a 2-Gy LDR exposure (delivered over 21 h) were significantly higher than those seen in 2-Gy HDR-treated cells in which a 21-h time period was allowed between the HDR IR exposure and the assay for γ-H2AX levels (data not shown). Furthermore, the
levels of γ-h2AX observed in LDR-treated cells were equivalent to those seen in HDR-treated cells when ATM was preactivated prior to the protracted LDR radiation exposures (Fig. 5, A and B). These data therefore strongly argue that the reduced levels of γ-h2AX seen in LDR-treated cells are not caused simply by dephosphorylation that occurs over the LDR irradiation time period.

The important role of ATM in cellular responses to LDR IR exposures is more fully supported by the observation that reduced clonogenic capacity and H2AX activation seen in LDR-treated cells were shown to be similar to that seen in HDR-treated cells in which ATM expression was severely reduced (Fig. 6, A and B). This was also noted in cells that were pretreated with the phosphatidylinositol 3-kinase inhibitor wortmannin (data not shown). Taken together, these data strongly argue that the increased cell killing (inverse dose rate effects) and reduced γ-h2AX levels following LDR IR exposures are primarily a consequence of reduced ATM activation. This hypothesis is in accordance with the finding that cells lacking ATM do not show inverse dose rate effects (34).

It is important to note that not all reduced dose rates result in increased cell death and reduced ATM and H2AX activation. Previous work (36, 37) in our laboratory has shown that the clonogenic survival of DU145 cells exposed to LDRs of 25 cGy/h is not significantly different from HDR-treated cells (data not shown). Furthermore, cells exposed to 2 Gy of IR delivered at the dose rate of 25 cGy/h did not show a reduction in the levels of activated ATM or H2AX compared with HDR-treated cells (data not shown), arguing that a dose rate threshold may exist for the activation of ATM and subsequent DNA damage responses. This threshold may be similar to the total IR dose threshold observed previously (8) for the activation of ATM. We were therefore interested to see whether even lower dose rates would result in increased amounts of cell killing as a result of further reductions in ATM activation. To achieve this, we exposed DU145 cells to the VLD of just 2 cGy/h, which produces only ~1 DNA DSB/h, and compared clonogenic survival with the activation of ATM and H2AX. We observed a much reduced clonogenic capacity in cells treated at the VLDR compared with clonogenic survival seen in HDR-treated cells (data not shown), suggesting that a dose rate threshold may exist for the activation of ATM and subsequent DNA damage responses. Thus, these novel findings may aid in further understanding the early cellular DNA damage response mechanisms to low level DNA damage, and the evasion of such mechanisms may be exploited for therapeutic benefit.

Acknowledgments—We thank Dr. Michael Kastan for providing the ATM antibody and Drs. Donald Coffey, John Isaacs, Fred Bunz, Brian Marples, and Janet Cronshaw for insightful discussions during the preparation of the manuscript.

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