Distinct Roles of Ape1 Protein, an Enzyme Involved in DNA Repair, in High or Low Linear Energy Transfer Ionizing Radiation-induced Cell Killing*

Received for publication, August 14, 2014, and in revised form, September 9, 2014; Published, JBC Papers in Press, September 10, 2014; DOI 10.1074/jbc.M114.604959

Hongyan Wang, Xiang Wang, Guangnan Chen, Xiangming Zhang, Xiaobing Tang, Dongkyoo Park, Francis A. Cucinotta, David S. Yu, Xingming Deng, William S. Dynan, Paul W. Doetsch, and Ya Wang

From the Department of Radiation Oncology, Emory University School of Medicine and Winship Cancer Institute of Emory University, Atlanta, Georgia 30322 and the Department of Health Physics and Diagnostic Sciences, University of Nevada, Las Vegas, Nevada 89154

Background: High LET radiation-induced DNA DSBs interfere with only NHEJ.

Results: Ape1 enzyme modification affects cell sensitivity to high LET but not to low LET radiation.

Conclusions: Ape1 promotes processing of clustered DNA damage into DSBs in high LET-irradiated cells.

Significance: This work presents new direction to develop approaches for either protecting astrocytes against radiation-induced base damage, which contributes to the higher RBE of high LET radiation, or repairing clustered DNA damage directly produced by high LET radiation, the latter of which Ape1 is involved in the generation of abasic sites. Here we demonstrate that Ape1, an enzyme required for processing apurinic/apyrimidinic sites, is also involved in the generation of clustered DNA damage. Additionally, we demonstrate that Ape1 promotes processing of clustered DNA damage into DSBs in high LET-irradiated cells.

This article has been withdrawn by Guangnan Chen, Dongkyoo Park, Francis A. Cucinotta, David S. Yu, Xingming Deng, William S. Dynan, Paul W. Doetsch, and Ya Wang. Hongyan Wang, Xiang Wang, Xiangming Zhang, and Xiaobing Tang could not be reached. The last two lanes of the actin immunoblot were reused in the last two lanes of the actin immunoblot in Fig. 1C. In Fig. 2A, the γ-H2AX and the merge with DAPI images for no IR treatment do not match. In Fig. 3A, lanes 3 and 4 of the γ-H2AX immunoblot were reused in lanes 7 and 8, and lanes 5 and 6 of the H2A immunoblot were reused in lanes 7 and 8. In Fig. 3B, lanes 5 and 6 of the H2A immunoblot were reused in lanes 7 and 8. In Fig. 3C, lanes 5 and 6 of the γ-H2AX immunoblot were reused in lanes 7 and 8. Additionally, lanes 1 and 2 of the H2A immunoblot were reused in lanes 3 and 4. In Fig. 3D, lanes 1 and 2 of the Mre11 immunoblot from lysates were reused in lanes 4 and 5. In the γ-H2AX immunoblot, lane 3 was reused in lane 7, and lane 4 was reused in lanes 6 and 8. Also in the H2A immunoblot, lanes 1 and 2 were reused in lanes 3 and 4. In Fig. 4B, lanes 2 and 6 of the Mre11 immunoblot from Ogg1−/− cells are the same. In the Ape1 immunoblot in Fig. 5C, lanes 3 and 5 are the same and lanes 6-8 are the same. Also, in the actin immunoblot, lanes 2 and 4 are the same.

NHEJ pathway of DNA DSB repair (8, 9). However, the 1.8-fold increase in small DNA fragments in high LET-irradiated cells versus low LET-irradiated cells (8) is insufficient to fully explain an RBE of 2–6 in mammalian cells. In looking for additional mechanisms, we considered the possibility of enzymatic modification at the clustered DNA damage sites because the enzymatic modification contributes to IR-induced total DSBs (11), and clustered DNA damage is more prevalent in high LET- than in low LET-irradiated cells (12, 13).
Ape1 Contributes to RBE on Cell Killing

The concept of clustered DNA damage was introduced by Goodhead et al. (14) and Ward (15), who proposed that passage of a radiation track through the nucleus results in localized clusters of single strand breaks, DSBs, and oxidized bases (16, 17). Repair of base damage is initiated by glycosylase (mainly Ogg1)-induced apurinic/apyrimidinic (AP) sites that are then cleaved by AP endonuclease (Ape1). Ape1 is an essential gene in mammalian cells and plays a protective role in low LET-irradiated cells (18, 19), although it can also generate DSBs by dual cutting of closely opposed AP sites (19, 20). Therefore, processing of clustered damage sites by Ogg1 and Ape1 may easily produce small DNA fragments. Because no practical physical materials can efficiently shield cells from high LET radiation, the investigation into whether enzyme modification of clustered damage sites was 40 bp. Materials can efficiently shield cells from high LET radiation, the investigation into whether enzyme modification of clustered damage sites could elucidate ways to sensitize tumor cells to high LET radiotherapy.

EXPERIMENTAL PROCEDURE

Plasmid Construction—The Ogg1 cDNA was cloned with the proper primers (Table 1) from C57BL/6J total RNA based on the reference transcript sequence (NMUST00000032406). The 1.1-kb RT-PCR product was recovered and inserted into the EcoRI and BglII sites of the pCMV-HA expression vector (purchased from Clontech). The wild type, enzymatically overactivated or inactivated human Ape1 was amplified by PCR using plasmid pCMV6-XL5 hAPEX1 (purchased from Origene Inc.) as template with proper primers (Table 1). The wild type, enzymatically overactivated or inactivated human Ape1 was amplified by PCR using plasmid pCMV6-kan.neo mAPEX1 (purchased from Origene Inc.) as template with proper primers (Table 1). The PCR products were recovered and inserted into the EcoRI and KpnI sites of the pCMV-HA expression vector. The pDRI-GFP plasmid was originally designed by Jasin and co-workers (21) for an HRR assay modified by inserting a second I-SceI site (5’-TAGGGATAACAGGGTAT-3’) upstream of the original I-SceI site (using a second, C-terminal truncated GFP as a donor template). The distance between the two I-SceI sites was 40 bp.

Cell Lines, Transfection, and Reagents—The mouse embryonic fibroblasts (MEFs), including Ku80−/−, Ku80+/+ (NHEJ-deficient) (8), Ogg1+/+ and Ogg1−/− cell lines (18), and transformed human fibroblast cell lines, including MRC5SV (wild type) and 180BRM (ligase IV mutant, NHEJ-deficient) cells (22–24), were used in this study. The cells were transfected with the wild type human or mouse Ape1 plasmid (purchased from Origene Inc.) using lipofectamine (Santa Cruz Biotechnology). At 30–40 h after transfection, the cells were collected for further experiments. The anti-Ogg1 antibody (catalog no. NB100-106) was purchased from Novus Biologicals Inc., and the anti-Mre11 antibody (catalog no. M226) was purchased from Santa Cruz Biotechnology. The anti-H2AX antibody (catalog no. 07–518) was purchased from Millipore Inc.

Irradiation—High LET IR was carried out using an x-ray generator (RAD 320, Precision X-ray, North Branford, CT; 320 kV, 2-mm aluminum filtration) in our laboratory. High LET IR was carried out using an alternating gradient synchrotron to generate 600 MeV/nucleon. 56Fe ions (180 keV/m; range in water, 27 cm; beam area, 20 × 20 cm; uniformity, ±5%) at Brookhaven National Laboratory. The dose rates for both high LET IR and low LET IR were ~1 Gy/min.

Cell Sensitivity to IR—Cell sensitivity to IR was examined using a clonogenic assay as described previously (8).

 Detecting Mre11 in Chromatin DNA-Protein Complexes—The experimental procedure was similar to that described previously (9).

 Detecting γ-H2AX Foci Assay—This assay was performed as we described previously (25).

 Detecting γ-H2AX and H2A Levels in Cells—The experimental procedure was as described in our previous publication (26).

 Detecting DNA in the Mre11-DNA Complex—The experimental procedure for detecting DNA in Mre11 complexes is similar to that described previously (9). Here, we summarize it in Fig. 4A. Briefly, 107 cells were used for paraformaldehyde cross-linking, and the cells were sonicated to produce DNA fragments of <1000 bp, immunoprecipitated using an anti-Mre11 antibody, and sonicated again. A small portion of each sample was reserved for detecting the Mre11 level. The DNA in the remainder was labeled with [γ-32P]ATP using polynucleotid kinase. Following exhaustive protease digestion, DNA fragments were analyzed by native PAGE. DNA signals were...
detected and analyzed by a Typhoon 9210 (GE Healthcare) Phospho Imager with ImageQuant software.

**Ape1 Activity Detection**—The Ape1 endonuclease activity assay method was modified based on the published method (27). For Ape1 substrate preparation, two molecular beacons (APSUB and APCTRL) were designed. APSUB is the Ape1 substrate with an abasic site, and APCTRL is the control beacon without an abasic site. Both are labeled with 6-FAM fluorophore at the 5'-end and Dabcyl quencher at the 3'-end. The sequences are as follows (internal 1'-2'-dideoxyribose spacer is an abasic spacer): APSUB, 5'-6-FAM-CCACT/idsp/TTGAAT-TGACACCGCAGATTCAGACTGG-Dabcyl-3'; APCTRL, 5'-6-FAM-CCACTCTTGAATTGACACGC- CATGCTGATCATTCAAGAG-TGG-Dabcyl-3'.

When annealed, the beacon formed a stem-loop structure. The lyophilized DNA oligonucleotides were dissolved in oligonucleotide dilution buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and concentrated to a Molecular Beacon stock solution at 40 μM. A 200 nM Molecular Beacon working solution was prepared using base excision repair reaction buffer (25 mM HEPES-KOH, 150 mM KCl, 0.5 mM EDTA, 1% glycerol, 0.5 mM DTT). After boiling for 3 min and cooling down slowly, APSUB...
Ape1 contributes to RBE on cell killing

or APCTRL in the working solution was allowed to anneal, and the melting curve quality was tested. The substrates were then ready for an Ape1 activity measurement. For nuclear extract preparation, MEF cells were transfected with siRNA against Ape1, control vector, wild type mApe1, mApe1-R176A, or mApe1-D209A expression plasmids in the pCMV-HA expression vector (Clontech, PT3283-5) using Lipofectamine 3000 (Invitrogen). At 24 h after transfection, cells were irradiated and put back in a 37 °C incubator. The cells were collected at 1 h after IR, and the nuclear extracts were prepared using an NE-PER kit (Thermo Scientific (Pierce)) and then dialyzed in the buffer (50 mM HEPES-KOH, 100 mM KCl, 0.5 mM EDTA, 20% glycerol, 1 mM DTT) for 4.5 h with three buffer changes. 30 µg of nuclear protein from each sample was mixed with 3.2 pmol of APSUB or APCTRL in reaction buffer at 37 °C for 60 min. The 6-FAM fluorescence was detected in a Fast 7500 real-time PCR machine. The initial rates of fluorescence in each well were determined to represent the Ape1 endonuclease activity.

**RESULTS**

**HRR Detection**—The digestion efficiencies of I-SceI for the plasmid were examined *in vitro* using I-SceI enzyme (purchased from New England Biolabs). Briefly, 250 ng of plasmid were digested with 2 units of I-SceI for 30 min at room temperature. 10 ng of the digested products were used as the template for PCR, and the undigested plasmid was used as a control. The PCRs were run for 15 cycles with proper primers (Table 1) and were used to check the downstream I-SceI site, which generated a 694-bp PCR product when the I-SceI site was within the total plasmid DNA (including upstream digestion efficiency was analyzed by a ratio of digested DNA to total plasmid DNA (including upstream). No digestion efficiency was detected with nuclear extract that masked the enzymatic digestion effects of Ape1 (19).

**Up-regulation of Ape1 Promotes High LET IR-induced Cell Sensitization**—To test the hypothesis that Ape1 may affect cell survival through enzymatic digestion, and high LET radiation, we first chose the approach of enhancing the Ape1 function by up-regulating the normal Ape1 protein because the Ape1 knockdown approach generated side effects on cell survival that masked the enzymatic digestion effects of Ape1 (19). Transiently transfecting a wild type Ape1 cDNA vector into wild type or Ku80−/− cells (NHEJ-deficient MEF lines (28)) increased expression of Ape1 2–3-fold relative to cells transfected with a control vector (Fig. 1A). Under such conditions, the up-regulated Ape1 did not affect the sensitivity of the cells to low LET IR but significantly sensitized the wild type and Ku80−/− cells to high LET IR (Fig. 1B). To further study whether the sensitization of Ape1-overexpressing cells to high LET IR was due to the AP site digestion function of Ape1, we examined the effects of Ape1 in Ogg1−/− MEFs, which are deficient in the generation of AP sites (29). Overexpression of Ape1 in Ogg1−/− cells (Fig. 1C) did not change the cell sensitivity to either low or high LET IR (Fig. 1D), which is different from the results obtained from wild type or NHEJ-deficient MEF cells with DNA glycosylase activity (Fig. 1B). Re-expression of Ogg1 in the Ogg1−/− cells (Fig. 1C) restored the overexpression of Ape1-induced cell sensitization to high LET radiation (Fig. 1D).

Similar results were also obtained from human wild type (MRC5SV) and NHEJ-deficient 180BRM cells (Fig. 1, E and F). When Ogg1 was knocked down in MRC5SV cells, the Ape1 overexpression-induced radiosensitization effect disappeared (Fig. 1, E and F), confirming that the Ape1 overexpression-induced radiosensitization depends on Ogg1. Notably, without overexpression of Ape1, the RBE of cell killing is 1 for NHEJ-deficient MEF (Ku80−/−) and human (180BRM) cells (Fig. 1G) (8), but overexpression of Ape1 results in the RBE increasing to 2 (Fig. 1G), suggesting that additional DNA DSBs occurred in these Ape1-overexpressing cells as well.
Up-regulation of Ape1 Produces More Unrepaired DNA DSBs in High LET- than in Low LET-irradiated Cells—To demonstrate that up-regulation of Ape1 generated more small DNA fragments in high LET-irradiated cells than in low LET-irradiated cells, we transfected MEF cells with control plasmid or the plasmid encoding Ape1 at 1 h after exposure to 10 Gy of low or high LET IR. The data represent the mean ± S.D. from three independent experiments; *, *p < 0.05. B, the levels of Mre11 or H2A in whole cell lysates (WC lysates) were examined in NHEJ-deficient (Ku80−/−) MEF cells transfected with control plasmid or the plasmid encoding Ape1 at 1 h after exposure to high LET or low LET radiation (Fig. 2, A and B). Notably, overexpression of Ape1 in Ogg1−/− MEF cells made no difference in the number of γ-H2AX foci after exposure to high LET or low LET radiation (Fig. 2C). Re-expression of Ogg1 restored the difference in the number of γ-H2AX foci-positive cells with and without Ape1 overexpression after exposure to high LET radiation (Fig. 2C). Similarly, overexpression of Ape1 in human cells (wild type MRC5SV or NHEJ−/− deficient 180BRM) increased the remaining γ-H2AX foci-positive cells at 4 or 24 h after exposure to high LET IR but did not affect those exposed to low LET IR (Fig. 2, D and E). These data match the survival data (Fig. 1) and indicate that the Ape1 up-regulation results in additional DNA DSBs only in high LET-irradiated cells when Ogg1 is present. These results also suggest that Ape1 can generate small DNA fragments in clusters of damaged DNA by cleaving closely opposed Ogg1-induced AP sites.

Ape1 Contributes to RBE on Cell Killing

![Figure 3](Image)

**FIGURE 3.** Up-regulation of Ape1 results in more Mre11 bound to chromatin DNA in high LET- than in Low LET-irradiated cells. A, the levels of Mre11 or γ-H2AX associated with chromatin (Chr bound) were examined in the WT MEF cells transfected with control plasmid or the plasmid encoding Ape1 at 1 h after exposure to 10 Gy of low or high LET IR. The levels of Mre11 or H2A in whole cell lysates (WC lysates) were examined in NHEJ-deficient (Ku80−/−) MEF cells transfected with control plasmid or the plasmid encoding Ape1 at 1 h after exposure to 10 Gy of low or high LET IR. The data represent the mean ± S.D. from three independent experiments; *, *p < 0.05. B, the levels of Mre11 or H2A in whole cell lysates (WC lysates) were examined in the WT MEF cells transfected with control plasmid or the plasmid encoding Ape1 at 1 h after exposure to 10 Gy of low or high LET IR. The data represent the mean ± S.D. from three independent experiments. *, *p < 0.05. C, the levels of Mre11 or γ-H2AX associated with chromatin were examined in Ogg1−/− MEF cells transfected with control plasmid or the plasmid encoding Ape1 at 1 h after exposure to 10 Gy of low or high LET IR. The data represent the mean ± S.D. from three independent experiments. *, *p < 0.05.

Up-regulation of Ape1 in cells with Ogg1−/− resulted in almost 100% of the cells having γ-H2AX foci at 4 or 24 h after exposure to high LET radia-
tion resulted in almost 100% of the cells having γ-H2AX signals (Fig. 2). The γ-H2AX foci were more plentiful in wild type MEF (Ku80+/+) or NHEJ-deficient MEF (Ku80−/−) cells with Ape1 overexpression than in the cells transfected with control vectors at 4 and 24 h after high LET IR, but they did not show a difference in low LET-irradiated cells (Fig. 2, A and B). Notably, overexpression of Ape1 in Ogg1−/− MEF cells made no difference in the number of γ-H2AX foci after exposure to high LET or low LET radiation (Fig. 2C). Re-expression of Ogg1 restored the difference in the number of γ-H2AX foci-positive cells with and without Ape1 overexpression after exposure to high LET radiation (Fig. 2C). Similarly, overexpression of Ape1 in human cells (wild type MRC5SV or NHEJ−/− deficient 180BRM) increased the remaining γ-H2AX foci-positive cells at 4 or 24 h after exposure to high LET IR but did not affect those exposed to low LET IR (Fig. 2, D and E). These data match the survival data (Fig. 1) and indicate that the Ape1 up-regulation results in additional DNA DSBs only in high LET-irradiated cells when Ogg1 is present. These results also suggest that Ape1 can generate small DNA fragments in clusters of damaged DNA by cleaving closely opposed Ogg1-induced AP sites.
increased amount of Mre11 bound to chromatin in high LET-irradiated cells (Fig. 3D). These results suggest that overexpression of Ape1 generated additional DNA fragments of 150 bp in high LET-irradiated cells dependent on Ogg1.

Next, to confirm that overexpression of Ape1-induced increased Mre11 bound to chromatin is due to increased amounts of DNADSBs, we examined the yield of DNA fragments bound to Mre11 in the immunoprecipitated Mre11-DNA complex at 1 h after exposure to high LET radiation (Fig. 4A). Once again, overexpression of Ape1 did not change the amount of DNA bound by Mre11 in Ogg1−/− cells at 1 h after exposure to high LET IR (Fig. 4B and C), but re-expressing Ogg1 restored the increase in DNA bound to Mre11 in the high LET-irradiated cells (Fig. 4B and C). Combining these results shown in Figs. 2 and 3, we can conclude that up-regulation of Ape1 sensitized cells to high LET radiation cells by producing additional DNA fragments via digestion of DNA at Ogg1-dependent AP sites. These results also suggest that under physiological conditions, Ape1 may contribute to a higher RBE of high LET IR in cell killing.

**Ape1 Contributes to RBE on Cell Killing**

To directly demonstrate whether Ape1 contributes to high LET IR-induced RBE in cell killing—To directly demonstrate whether Ape1 contributes to high LET IR-induced RBE in cell killing at physiological levels, we generated three vectors that encode three different HA-tagged Ape1 constructs and are resistant to siRNA (Fig. 5A): wild type, overactivated Ape1, and inactivated Ape1. The overactivated Ape1 was generated by mutating amino acid 176 from Arg to Ala (34), and the inactivated Ape1 was generated by mutating amino acid 209 from Asp to Ala (35) (Fig. 5B). When the endogenous Ape1 was knocked down with the specific siRNA, the vector encoding HA-Ape1 was expressed in the cells (Fig. 5C). The Ape1 enzyme activities in these cells were verified (Fig. 5D). Notably, compared with cells expressed with wild type Ape1, cells expressed with Ape1 inactivation were more resistant to high LET IR, and cells expressed with Ape1 overactivation were more sensitive to high LET IR (Fig. 5E); however, there was no difference in the sensitivity of these cells to low LET IR (Fig. 5E). These results demonstrate that the enzymatic activity of Ape1 plays an important role in high LET IR-induced cell killing, suggesting that at a physiological level, Ape1 can contribute to the RBE on high LET radiation-induced cell killing.

Combining the 1.8-fold greater amount of small DNA fragments generated directly by high LET IR when compared with low LET IR (8) and the subsequent Ape1-generated DNA DSBs at the clustered DNA damage sites, we can better explain the RBE, 2–6, for general cell survival. It is believed that enzymatic modification contributes to 30–50% of IR-induced total DSBs (11), and thus the initial 1.8-fold more small DNA fragments generated by high LET radiation (8) would increase after Ape1
functions in high LET-irradiated cells, which results in more cell killing and a higher RBE.

Small DNA Fragments That Affect NHEJ Do Not Affect HRR—The fact that RBE in cell killing is equivalent for low and high LET radiation in NHEJ-deficient cells indicates that high LET radiation only interferes with NHEJ and not with HRR. This conclusion has been verified by our group (8, 9, 36) and another group (10). The mechanism involves high LET IR-generated small DNA fragments (≤40 bp) whose size prevents Ku from simultaneously binding to the two ends of one DNA fragment to initiate efficient NHEJ (8). We have also shown that small DNA fragments (≤40 bp) do not affect the DNA binding efficiency of Mre11 (an initial step for HRR) (9, 36). These differences arise from the different structures, DNA binding characteristics, and functions of the two proteins: Ku (32, 37) and Mre11 (30, 38).

To provide direct evidence that small DNA fragments (≤40 bp) do not affect HRR efficiency in vivo, we used the I-SceI-HRR-reporter system by modifying the pDR-GFP construct that was originally designed by Jasin and co-workers (21). The modified plasmid contains two sites for I-SceI digestion separated by 40 nucleotides within the GFP expression frame (Fig. 6A). We first compared the I-SceI digestion efficiency for the plasmids containing one digestion site and two digestion sites in vitro by PCR to amplify the digestion products with specific primers. The results showed that the I-SceI enzyme requires ≥20 bp at each side of the digestion site for efficient digestion. After confirming that the plasmids had integrated into transfected human 293FT cells using the same primers to amplify PCR products from the genomic DNA, we measured the HRR efficiency by transfecting I-SceI in these cells. The results showed that the difference in HRR efficiency (≥3-fold difference) in the cells containing the plasmid DNA with one I-SceI-digestion site and those with two I-SceI-digestion sites (Fig. 6C) was similar to that in the original comparison of I-SceI digestion efficiency (≥3-fold difference). These results indicate that a 40-bp DNA fragment does not decrease HRR efficiency, although DNA
fragments of this size affect efficient NHEJ. These results strongly suggest that the small DNA fragments (≤40 bp) generated by either heavy ions directly penetrating a nucleosome or consequent Ape1 digestion within clustered DNA damage in high LET-irradiated cells do not affect HRR (Fig. 6D).

**DISCUSSION**

Ape1 was independently identified as a reductive activator of the AP-1 (c-Jun/Fos heterodimer) transcription factor and named redox effector factor 1 (Ref-1) (39). Ape1 is involved in both the base excision repair pathway and the regulation of gene expression as a redox co-activator of different transcription factors, such as early growth response protein-1 (Egr-1), p53, HIF1-α, and AP-1 (40, 41). These activities are controlled by two functionally distinct domains; the N terminus is mainly devoted to the gene regulation, whereas the C terminus has the enzymatic activity on the AP sites of DNA (34, 42).

The survival threat of knockout of Ape1 to mice (43) or knockdown of Ape1 to cells (19) may depend mainly on the gene regulation function of Ape1 because Ogg1 (the main enzyme to generate the AP site in base excision repair) deficiency did not affect mouse survival (29). Up- or down-regulating the Ape1 activity without IR did not affect cell sensitivity to low IR induced killing, which also supports the above prediction. In addition, exogenous expression of enzyme-inactivated Ape1 as compared with wild type Ape1 when the endogenous Ape1 in the cells was knocked down decreased IR-induced cell killing (Fig. 5E) and provides additional evidence to suggest that the survival threat of knockout/knockdown of Ape1 depends mainly on the endogenous Ape1 in the cells was knocked down decreased IR-induced cell killing (Fig. 5E) and provides additional evidence to suggest that the survival threat of knockout/knockdown of Ape1 depends mainly on the endogenous Ape1. Modifying the Ape1 activity primarily affects cell sensitivity to high LET radiation but does not affect cell sensitivity to low LET radiation, which is mainly because of the difference in the structure of damaged DNA generated by high LET IR and low LET IR. High LET radiation with the dense ionizing events generates more cluster DNA damage than low LET radiation (13). At clustered DNA damage sites, it is much easier for Ape1 to generate small DNA fragments (Fig. 6D).

The same amount of ionizing events occur in the same doses of high LET- and low LET-irradiated cells, which results in the
Ape1 Contributes to RBE on Cell Killing

same yield of DNA DSBs. However, it is known that high LET does kill more wild type, HRR-deficient cells than low LET IR but not the Ku-dependent NHEJ-deficient cells (6–8, 10), indicating that high LET IR interferes with only Ku-dependent NHEJ. We believe that the small DNA fragment model is one of the major reasons for the phenotypes because there are no other models supported by convincing evidence to explain why high LET IR interferes with only the Ku-dependent NHEJ. One question concerning the small DNA fragment model is why the damaged DNAs do not simply lose the small fragments and directly join the remaining ends. In fact, the radiation-induced small DNA fragments are very likely lost if they are located between nucleosomes without histone attachments. However, histone attachments protect the damaged DNAs from losing small fragments when they are located within nucleosomes. The observation of more DNA fragments (≤40 bp) in high LET-irradiated cells than in low LET-irradiated cells (8) and the RBE of 1 for NHEJ-deficient cells strongly support this explanation. Based on our results, it would be possible to reduce high LET IR-induced damage in normal cells by down-regulating the Ape1 activity or to sensitize tumor cells to high LET radiotherapy by up-regulating the Ape1 activity in the near future.

Taken together, these results reveal that the base excision repair function of Ape1 plays different roles in low LET- and high LET-irradiated cells due to the dense clustered DNA damage generated by high LET radiation. This new discovery could contribute to improve either high LET radiation or high LET radiotherapy.

Acknowledgments—We thank Drs. Mitra, Li, Malaise, and Jasin for high LET radiotherapy.

REFERENCES

1. Zeitlin, C., Hassler, D. M., Cuchiaro, J., Broughton, B., Wimmer-Schweingruber, R. F., Brinza, D., Grasser, G., Wurtele, G., Böttcher, S., Böhm, E., Burmeister, S., Guo, J., Köhler, J., Martin, C., Posner, A., Rafkin, S., and Reitz, G. (2013) Measurements of energetic particle radiation in transit to mars on the Mars Science Laboratory, Science \textbf{340}, 1080–1084

2. Aufderheide, E., Rink, H., Hieber, L., and Kraft, G. (1987) Heavy ion effects on cellular DNA: strand break induction and repair in cultured diploid lens epithelial cells. \textit{Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.} \textbf{51}, 779–790

3. Rydberg, B., Löbrich, M., and Cooper, P. K. (1994) DNA double-strand breaks induced by high-energy neon and iron ions in human fibroblasts. \textit{I. Pulsed-field gel electrophoresis method}. \textit{Radiat. Res.} \textbf{139}, 133–141

4. Rothkamm, K., Krüger, I., Thompson, L. H., and Löbrich, M. (2003) Pathways of DNA double-strand break repair during the mammalian cell cycle. \textit{Mol. Cell. Biol.} \textbf{23}, 5706–5715

5. Khanna, K. K., and Jackson, S. P. (2001) DNA double-strand breaks: signaling, repair and the cancer connection. \textit{Nat. Genet.} \textbf{27}, 247–254

6. Lind, B. K., Persson, L. M., Edgren, M. R., Hedlöf, I., and Brahme, A. (2003) Repairable: conditionally repairable damage model based on dual Poisson processes. \textit{Radiat. Res.} \textbf{160}, 366–375

7. Okayasu, R., Okada, M., Okabe, A., Noguchi, M., Takakura, K., and Taka-hashi, S. (2006) Repair of DNA damage induced by accelerated heavy ions in mammalian cells proficient and deficient in the non-homologous end-joining pathway. \textit{Radiat. Res.} \textbf{165}, 59–67

8. Wang, H., Wang, X., Zhang, P., and Wang, Y. (2008) The Ku-dependent non-homologous end-joining but not other repair pathway is inhibited by high linear energy transfer ionizing radiation. \textit{DNA Repair} \textbf{7}, 725–733

9. Wang, H., Zhang, X., Wang, P., Yu, X., Essers, J., Chen, D., Kanaar, R., Takeda, S., and Wang, Y. (2010) Characteristics of DNA-binding proteins determine the biological sensitivity to high-linear energy transfer radiation. \textit{Nucleic Acids Res.} \textbf{38}, 3245–3251

10. Zafar, F., Seidler, S. B., Kronenberg, A., Schild, D., and Wiese, C. (2010) Homologous recombination contributes to the repair of DNA double-strand breaks induced by high-energy iron ions. \textit{Radiat. Res.} \textbf{173}, 27–39

11. Georgakilas, A. G., Bennett, P. V., Wilson, D. M., 3rd, and Sutherland, B. M. (2004) Processing of bistranded abasic DNA clusters in γ-irradiated human hematopoietic cells. \textit{Nucleic Acids Res.} \textbf{32}, 5609–5620

12. Brenner, D. J., and Ward, J. F. (1992) Constraints on energy deposition and target size of multiply damaged sites associated with DNA double-strand breaks. \textit{Int. J. Radiat. Biol.} \textbf{61}, 737–748

13. Asaithamby, A., Hu, B., and Chen, D. J. (2011) Unrepaired clustered DNA lesions induce chromosome breakage in human cells. \textit{Proc. Natl. Acad. Sci. U.S.A.} \textbf{108}, 8293–8298

14. Goodhead, D. T., Munson, R. J., Thacker, J., and Cox, R. (1980) Mutation and inactivation of cultured mammalian cells exposed to beams of accelerated heavy ions. \textit{IV. Biophysical interpretation}. \textit{Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.} \textbf{37}, 135–167

15. Ward, J. F. (1981) Some biological consequences of the spatial distribution of ionizing radiation and free radicals. \textit{Radiat. Res.} \textbf{86}, 185–195

16. Sutherland, B. M., Bennett, P. V., Degtereva, O., and Laval, J. (2000) Clustering of DNA double-strand breaks in DNA and in human cells by low dose X-rays. \textit{Int. J. Radiat. Biol. U.S.A.} \textbf{97}, 103–108

17. Wang, H., Zhang, X., Wang, P., Yu, X., Essers, J., Chen, D., Kanaar, R., Nussenzweig, M. C., and Li, G. C. (1996) Requirement for Ku80 in growth of human hematopoietic cells. \textit{Mol. Cell. Biol.} \textbf{16}, 699–702

18. Ramana, C. V., Boldogh, I., Izumi, T., and Mitra, S. (1998) Activation of APE1 by DNA damage in human cells by reactive oxygen species and its correlation with their adaptive response to genotoxicity of ionizing radiation. \textit{Proc. Natl. Acad. Sci. U.S.A.} \textbf{95}, 5061–5066

19. Bostick, B. (2011) Distinct roles of Ape1 protein in the repair of DNA damage induced by ionizing radiation or bleomycin. \textit{J. Biol. Chem.} \textbf{286}, 4968–4977

20. Malyarchuk, S., Castore, R., and Harrison, L. (2009) Ape1 can cleave complex clustered DNA lesions in cells. \textit{DNA Repair} \textbf{8}, 1343–1354

21. Pierce, A. J., Johnson, R. D., Thompson, L. H., and Jasin, M. (1999) XRC33 promotes homology-directed repair of DNA damage in mammalian cells. \textit{Genes Dev.} \textbf{13}, 2633–2638

22. Badie, C., Iliakis, G., Foray, N., Alsbeih, G., Pantellas, G. E., Okayasu, R., Cheong, N., Russell, N. S., Begg, A. C., Arlett, C. F., and Malaise, E. P. (1995) Defective repair of DNA double-strand breaks and chromosome damage in fibroblasts from a radiosensitive leukemia patient. \textit{Cancer Res.} \textbf{55}, 1232–1234

23. Riballo, E., Critchlow, S. E., Teo, S.-H., Doherty, A. J., Priestley, A., Broughton, B., Kysela, B., Beamish, H., Plowman, N., Arlett, C. F., Lehmann, A. R., Jackson, S. P., and Jego, P. A. (1999) Identification of a defect in DNA ligase IV in a radiosensitive leukemia patient. \textit{Curr. Biol.} \textbf{9}, 699–702

24. Wang, H., Zeng, Z.-C., Perrault, A. R., Cheng, X., Qin, W., and Iliakis, G. (2001) Genetic evidence for the involvement of DNA ligase IV in the DNA-PK-dependent pathway of non-homologous end joining in mammalian cells. \textit{Nucleic Acids Res.} \textbf{29}, 1653–1660

25. Zheng, Z., Ng, W. L., Zhang, X., Olson, J. J., Hao, C., Curran, W. J., and Wang, Y. (2012) RNA-mediated targeting of noncoding and coding sequences in DNA repair gene messages efficiently radiosensitizes human tumor cells. \textit{Cancer Res.} \textbf{72}, 1221–1228

26. Wang, H., Wang, M., Wang, H., Böcker, W., and Iliakis, G. (2005) Complex H2AX phosphorylation patterns by multiple kinases including ATM and DNA-PK in human cells exposed to ionizing radiation and treated with kinase inhibitors. \textit{J. Cell Physiol.} \textbf{202}, 492–502

27. Sviari, D., Vens, C., and Sobol, R. W. (2012) Quantitative, real-time analysis of base excision repair activity in cell lysates utilizing lesion-specific molecular beacon. \textit{J. Vis. Exp.} \textbf{10.3791/4168}

28. Nussenzwieg, A., Chen, C., da Costa Soares, V., Sanchez, M., Sokol, K., Nussenzwieg, M. C., and Li, G. C. (1996) Requirement for Ku80 in growth of...
and immunoglobulin V(D)J recombination. Nature 382, 551–555
29. Klungland, A., Rosewell, I., Hollenbach, S., Larsen, E., Daly, G., Epe, B., Seeberg, E., Lindahl, T., and Barnes, D. E. (1999) Accumulation of pre-mutagenic DNA lesions in mice defective in removal of oxidative base damage. Proc. Natl. Acad. Sci. U.S.A. 96, 13300–13305
30. Williams, R. S., Moncalian, G., Williams, J. S., Yamada, Y., Limbo, O., Shin, D. S., Grocock, L. M., Cahill, D., Hitomi, C., Guenther, G., Moiani, D., Carney, J. P., Russell, P., and Tainer, J. A. (2008) Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. Cell 135, 97–109
31. Buis, J., Wu, Y., Deng, Y., Leddon, J., Westfield, G., Eckersdorff, M., Sekiguchi, J. M., Chang, S., and Ferguson, D. O. (2008) Mre11 nuclease activity has essential roles in DNA repair and genomic stability distinct from ATM activation. Cell 135, 85–96
32. Walker, J. R., Corpina, R. A., and Goldberg, J. (2001) Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. Nature 412, 607–614
33. Dupré, A., Boyer-Chatenet, L., and Gautier, J. (2006) Two-step activation of ATM by DNA and the Mre11-Rad50-Nbs1 complex. Nat. Struct. Mol. Biol. 13, 451–457
34. Mol, C. D., Izumi, T., Mitra, S., and Tainer, J. A. (2000) DNA-bound structures and mutants reveal abasic DNA by APE1 DNA repair and coordination. Nature 403, 451–456
35. Erzberger, J. P., and Wilson, D. M., 3rd (1999) The role of Mg2+ and specific amino acid residues in the catalytic reaction of the major human abasic endonuclease: new insights from EDTA-resistant incision of acyclic abasic site analogs and site-directed mutagenesis. J. Mol. Biol. 290, 447–457
36. Wang, H., Liu, S., Zhang, P., Zhang, S., Naidu, M., Wang, H., and Wang, Y. (2009) S-phase cells are more sensitive to high-linear energy transfer radiation. Int. J. Radiat. Oncol. Biol. Phys. 74, 1236–1241
37. Yoo, S., and Dynan, W. (1999) Geometry of a complex formed by double strand break repair proteins at a single DNA end: recruitment of DNA-PKcs induces inward translocation of Ku protein. Nucleic Acids Res. 27, 4679–4686
38. Shibata, A., Moiani, D., Arvai, A. S., Perry, J., Harding, S. M., Genois, M.-M., Maity, R., van Rossum-Fikkert, S., Kertokalio, A., Romoli, F., Ismail, A., Ismail, E., Neale, M. J., Bristow, R. G., Masson, J.-Y., Wyman, C., Jeggo, P. A., and Tainer, J. A. (2014) DNA double-strand break repair pathway choice is directed by distinct MRE11 nuclease activities. Mol. Cell 53, 7–18
39. Xanthoudakis, S., and Curran, T. (1992) Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. EMBO J. 11, 653–665
40. Tell, G., Damante, G., Caldwell, D., and Kelley, M. R. (2005) The intracellular localization of APE1/Ref-1: more than a passive phenomenon? Antioxid. Redox Signal. 7, 367–384
41. Bhakat, K. K., Mantha, A. K., and Mitra, S. (2009) Transcriptional regulatory functions of mammalian AP-endonuclease (APE1/Ref-1), an essential multifunctional protein encoded by monoverlapping domains. Proc. Natl. Acad. Sci. U.S.A. 106, 20939–20944
42. Xanthoudakis, S., and Curran, T. (1996) The redox and DNA-repair activities of Ref-1 are encoded by monoverlapping domains. Proc. Natl. Acad. Sci. U.S.A. 93, 8919–8923