Radiation Resistance in Glioma Cells Determined by DNA Damage Repair Activity of Ape1/Ref-1

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Glioma/Lucanthone/Radiation tolerance/DNA Repair/DNA- (Apurinic or Apyrimidinic Site) lyase/ Ape1/Ref-1/APEX/HAP1.

Since radiation therapy remains a primary treatment modality for gliomas, the radioresistance of glioma cells and targets to modify their radiation tolerance are of significant interest. Human apurinic endonuclease 1 (Ape1, Ref-1, APEX, HAP1, AP endo) is a multifunctional protein involved in base excision repair of DNA and a redox-dependent transcriptional co-activator. This study investigated whether there is a direct relationship between Ape1 and radioresistance in glioma cells, employing the human U87 and U251 cell lines. U87 is intrinsically more radioresistant than U251, which is partly attributable to more cycling U251 cells found in G2/M, the most radiosensitive cell stage, while more U87 cells are found in S and G1, the more radioresistant cell stages. But observed radioresistance is also related to Ape1 activity. U87 has higher levels of Ape1 than does U251, as assessed by Western blot and enzyme activity assays (~1.5–2 fold higher in cycling cells, and ~10 fold higher at G2/M). A direct relationship was seen in cells transfected with CMV-Ape1 constructs; there was a dose-dependent relationship between increasing Ape1 overexpression and increasing radioresistance. Conversely, knock down by siRNA or by pharmacological down regulation of Ape1 resulted in decreased radioresistance. The inhibitors lucanthone and CRT004876 were employed, the former a thioxanthene previously under clinical evaluation as a radiosensitizer for brain tumors and the latter a more specific Ape1 inhibitor. These data suggest that Ape1 may be a useful target for modifying radiation tolerance.

INTRODUCTION

Radiation therapy continues to play a central role in the management of brain tumors.¹) Since tumor recurrences tend to appear within 2 cm of the original tumor and within the irradiated volume, one might have predicted that escalating the dose of radiation to high-grade gliomas would improve local control. But some recent clinical outcome studies failed to support this notion, although the doses were limited to a safe range for the patients. Nevertheless, the radioresistance of high-grade gliomas remains one of the reasons for the failure of treatment, which would otherwise require tumoricidal doses beyond the normal tissue tolerance and beyond safe clinical practice.²) Thus, determining the cellular, molecular and biochemical mechanisms underlying radiation resistance becomes critical for successful treatment of these tumors. For this purpose, glioma cell lines U87 and U251,³) were employed in this study.

Multiple factors limit the efficacy of radiation. Chief among them are changes in cell cycle, activation of oncogenes, and inactivation of tumor suppressor genes.⁴–⁶) Another important determinant of cellular radiosensitivity is DNA repair proficiency,⁷) however, there may be cell type specific effects. Glioma cell lines show lower total rejoining capacity than prostate, melanoma or squamous cell carcinoma even though they have similar radiosensitivities.⁷) Glioblastoma cells can survive high doses of radiation even when they contain high levels of unjoined or mis-rejoined DNA damage.⁸) Cell lines exhibit clonal variation in radiosensitivity and in the fidelity of DNA repair, and when this was studied in one glioma line,⁹) radiosensitivity was shown to be related to increased misrepair. Nuclear protein extracts derived from radiosensitive clones were less capable of correctly rejoining EcoRI-induced double strand breaks (DSB).
than were similar extracts from radioresistant clones. These clones did not vary significantly in proliferation rates, and variation of radiosensitivity in different stages of the cell cycle is a very well known phenomenon, thus the differences in radiosensitivity were more likely due to differences in DSB mis-rejoining frequencies. These studies imply differences in the levels of DNA repair enzymes or differences in repair pathways [base excision repair (BER), non-homologous end joining (NHEJ), homologous recombinational repair (HRR)] between two clones, which differ in radiosensitivity.

The question then arises, which kind of repair pathway or enzymes are operational in these radioresistant glioma cell lines? One candidate is Ape1, whose expression is very high in gliomas compared to other repair enzymes such as 8-oxoguanine glycosylase-1, O6-methylguanine-DNA methyltransferase, and methylpurine-DNA glycosylase. Ape1 is a multifunctional protein that acts: (i) as an endonuclease during the second step of the BER pathway, and is responsible for the repair of cellular alkylation and oxidative DNA damage; (ii) as a transcriptional co-activator which maintains several transcription factors in an active reduced state, which in turn regulate cellular processes such as apoptosis, proliferation, and differentiation; (iii) as a transcriptional co-activator by redox-independent mechanisms (iv) to control intracellular reactive oxygen species production by negatively regulating the activity of the Ras-related GTPases and very recently as an endoribonuclease capable of cleaving c-myc mRNA (for review, see e.g. Rothwell et al., Evans et al.). In addition to the designation of Ape1, apurinic/apyrimidinic (AP) endonuclease is also referred to as APEX nuclease and APEX endo; it is also known as redox effector factor-1 (Ref-1) and human AP endonuclease-1 (HAP1) – the latter not to be confused with huntingtin-associated protein also known as HAP1. Ape1 has two distinct functions (oxidative damage repair and gene regulation), which are both essential for cell survival in vivo and in vitro. Fung & Demple revealed that the DNA repair function of Ape1 is critical for cell viability – siRNA knock down suppressed cell proliferation and promoted apoptosis, but these effects were reversed by overexpression of the yeast Ape1 protein which lacks redox activity but shares the Ape1 DNA repair activity. The association of Ape1 and radiation and chemotherapy responses has been observed in medulloblastoma and primitive neuroectodermal tumors, and there is both evidence for and against a correlation between Ape1 levels and radioresistance in tumors. In cell culture studies, Ape1 contributes to glioma cell resistance to alkylating agents, and its endonuclease activity is increased by oxidative stress. Human glioma cell lines that show lower Ape1 expression had higher sensitivity to methyl methanesulfonate (MMS) and H2O2, the known inducers of AP sites and single-strand breaks in DNA. The overexpression of Ape1 in NT2 neural progenitor cells confers resistance to bleomycin and radiation. Recently, the radioresistance of prostate tumor cells was shown to decrease when Ape1 levels were down regulated by isoﬂavones, even in transfected cells overexpressing Ape1, and these findings were supported by in vivo experiments with mice harboring orthotopic prostate tumors.

Thus, while there is good reason to suspect it is the case, no reports to date have yet correlated Ape1 levels in glioma cells with radioresistance. Hence, we conducted experiments to determine if Ape1 expression in two glioma cell lines, U251 and U87, accounted for differences in their radioresistance. Since it was possible that the U87 and U251 glioma cell lines might show variation in radiation sensitivity due to their different intrinsic rates of proliferation, and since the expression of the Ape1 gene is coordinated with the cell cycle (Ape1 mRNA levels rise after the G1-S transition and peak in early to mid-S phase) we sought to analyze the radiation response of these cells during different stages of the cell cycle together with Ape1 activity and determine if this Ape1 expression was cell cycle-dependent or independent. Further, we employed lucanthone, a specific inhibitor of Ape1 known to generate AP sites which are substrates for Ape1. Lucanthone was originally known as an anti-schistosomal agent, but was determined to be a DNA intercalating agent and a topoisomerase inhibitor with cytostatic effects and therefore it and its structural analog, hycanthone, found their way into clinical trials as radiotherapy adjuvants. To further nail down the role of Ape1’s specific activity in radioresistance of these glioma cell lines, we employed another Ape1 specific inhibitor CRT0044876 that is known to directly bind the active site of Ape1. Finally, we were able to go beyond a simple correlation between Ape1 and radioresistance and show a causal relationship in experiments in which Ape1 was overexpressed in U251 cells.

**MATERIALS AND METHODS**

**Reagents**

U87-MG and U251-MG were a kind gift from Dr. Dennis Deen of UCSF. These cells were maintained in Earle’s Minimal Essential Medium with 2 mM L-Glutamine and 1.5 g/L sodium bicarbonate, supplemented with 10% fetal bovine serum (FBS), and were subcultured twice a week (1:3 for U87 and 1:5 for U251). Cell culture medium, FBS, and Lipofectamine 2000 transfection reagent were obtained from Invitrogen (Carlsbad, CA). Ape1 was detected using polyclonal anti-Ape1 (Trevigen, Gaithersburg, MD) and tubulin was detected using polyclonal anti-γ-tubulin (Sigma-Aldrich, St. Louis, MO). Lucanthone was obtained from Dr. Robert Bases, Albert Einstein School of Medicine, Bronx, NY and Dr. Michael Waring, Cambridge University, Cambridge, UK.
**Radiation exposure**

Both cell lines were seeded at 1.5 × 10^6 cells on 60 mm dishes in 3 ml of media. After 24 hrs, cultures were exposed to 50 kVp X-rays at doses ranging from 25 – 1500 cGy.

**Colony (clonogenic) survival**

Cells of varying concentrations were replated in 100 mm dishes, adjusted for plating efficiency and doubling time (660–2,150 cells/dish for U87; 250–12,000 cells/dish for U251) to obtain about 200 colonies. After 14 days without media change, the cultures were fixed with 70% ethanol in PBS and resuspended in 0.5 ml propidium iodide stain (25 μg/ml of propidium iodide and 100 μg/ml of RNAase A in PBS) and incubated at 37°C for 1 hr and then analyzed with Becton Dickerson FACScan/FACS Calibur instruments at the USB Flow Cytometry Core Facility (Stony Brook University, NY) at a setting of 20. The sonicates were then incubated at 37°C for 1 hr and then analyzed with Becton Dickerson FACScan/FACS Calibur instruments at the USB Flow Cytometry Core Facility (Stony Brook University, NY) at a setting of 20. The data were analyzed using ModFit software.

**Cell cycle**

The two cell lines were harvested after exposures and 1 × 10^6 cells were used for measuring the DNA content and the rest (0.5 × 10^6) were used for Ape1 protein and activity studies. Duplicate plates were pooled together. Those collected for 5–10 s in a 4 mM EDTA, 100 mM NaCl and 1 mM PMSF) and sonicated in 10 mM Thymidine for 48 h (S arrest), or 40 μg/ml of Nocodazole for 16 h (G2/M arrest). The cell cycle arrest was determined using the Bradford assay (BioRad, Hercules, CA) and the data were analyzed using ModFit software.

**Cell extract preparation**

0.5 – 1.5 × 10^6 cells were resuspended in 200 μl of ice-cold 1X cell extraction buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl and 1 mM PMSF) and sonicated for 5–10 s in a 4°C bath sonicator (Sonifier Cell Disruptor, Plainview, NY) at a setting of 20. The sonicates were then spun in a refrigerated micro centrifuge at 10,000 rpm for 5 min at 4°C. Soluble and insoluble fractions were collected (insoluble pellet was resuspended in 200 μl of cell extraction buffer) and assayed for Ape1 protein and enzyme activity as described below.

**SDS-PAGE and Western Blot**

Total protein concentration in cell extracts was determined using the Bradford assay (BioRad, Hercules, CA) and 25 μg of total protein from soluble or insoluble cell fractions were mixed with an equal volume of gel loading buffer (0.001% bromophenol blue, 4% SDS, 10% 2-ME, 20% glycerol, and 125 mM Tris pH 6.8) and denatured at 95°C for 5 min. This mixture was separated by SDS-PAGE (4% stacking, 7.5% resolving gel) in duplicate gels for 2–3 hr at 40 mA on a BioRad MiniPROTEAN II Electrophoresis Cell. To detect proteins, one gel was stained with Coomassie Brilliant Blue R-250. Proteins on the other unstained gel were transferred onto a trans-Blot nitrocellulose membrane (0.45 μm; BioRad) overnight at 4°C at 15 mA in standard Tris-Glycine buffer containing 20% ethanol. Membranes were probed using polyclonal anti-Ape1 or polyclonal anti-γ-tubulin at 1/1,000 dilution in TTBS (0.1% Tween 20 in TBS (pH 7.5)) with secondary, anti-rabbit HRP-conjugated antibody used at 1/30,000 dilution to detect Ape1 and γ-tubulin protein control. Chemiluminescence was developed using an ECL kit according to the manufacturer’s directions and detected by exposing the blot to Hyperfilm-ECL for 30 s–3 min (GE Healthcare Biosciences, Piscataway, NJ).

**Ape1 endonuclease activity**

Ape1 activity was determined using an assay that measures the conversion of plasmid DNA from supercoiled to relaxed form caused by incision at an abasic site. This assay will also detect AP lyase activity, however the total Ape1 enzyme activity predominantly measures the endonuclease activity. Since Chen et al. showed that the non-specificity of Ape1 endonuclease activity using depurinated plasmid DNA was only about 1-2% relative to their endonuclease activities in HeLa cell extracts, and since Siber et al. had shown that Ape1 activity is very elevated in human gliomas, we choose this assay for determining Ape1 activity in glioma cell extracts. Briefly, the substrate used was 200 ng of depurinated pUC18 DNA (prepared by incubating pUC18 DNA in 20 mM Na-citrate, 0.2 M NaCl, pH 5.0, 70°C for 12 min.) in 10 μl of 1X Ape Buffer containing 50 mM HEPES, 150 mM KCl, 5 mM MgCl2 and 100 μg/ml of BSA in presence of different concentrations of the cell extracts (1 μl containing 0-55 ng of total protein). Similar reactions were set up with untreated pUC18, which served as the internal control. This reaction mixture was incubated at 37°C for 15 min and the reaction was stopped by addition of alkaline stop mix (0.25% bromocresol green in 0.25 N NaOH, 30% (w/v) glycerol) and left at room temperature for 10-15 min and then the products were resolved on a 1% agarose gel (BioRad) overnight at 4°C in an electronic vertical system and area under the supercoiled and relaxed form caused by incision at an abasic site (4,31) This reaction mixture was incubated at 37°C for 5 min and the reaction was stopped by addition of alkaline stop mix (0.25% bromocresol green in 0.25 N NaOH, 30% (w/v) glycerol) and left at room temperature for 10-15 min and then the products were resolved on a 1% agarose gel (BioRad) overnight at 4°C in an electronic vertical system and area under the supercoiled and relaxed form was determined and ratio was determined for the ratio of relaxed/relaxed + supercoiled to calculate incisions per plasmid molecule and the resulting depurinated pUC18 had 1 AP site per molecule. Final calculations were done in femtomoles (fmol) of abasic sites incised /min/mg protein with normalization done using Pyruvate Kinase units (PKU) present in these extracts.
Ape1 over-expression studies

A pcMV-Ape1 plasmid\(^{18}\) was transfected into U251 cells using Lipofectamine 2000 as per manufacturer’s instructions followed by selection in G418. Briefly, 30 μg of pCMV-Ape1 DNA (or pCMV10 control plasmid) was added to 1.875 ml of X-VIVO 10 (BioWhittaker Speciality Media, FisherScientific) and vortexed. 75 μl of Lipofectamine 2000 was added to a separate sterile 15 ml tube containing 1.875 ml of X-VIVO 10 and vortexed. After 10 min at room temperature, the two tubes were combined, vortexed, and left at room temperature for another 30 min. The DNA/Lipofectamine 2000 mixture was then added drop wise to a T75 flask-containing U251 cells at 80% confluence maintained in antibiotic-free, high glucose DMEM supplemented with 10% heat-inactivated FBS. After 24 hr the culture media was replaced with fresh culture media containing 100 μg/ml of G418 for selection over 10 days. To obtain clonal cell populations, following selection, a single cell was deposited into individual wells of 96 well tissue culture plates containing DMEM with 10% FBS and 50 μg/ml G418 using a Becton Dickinson FACSAria cell sorter at the FACS Core Facility of the Feinstein Institute for Medical Research, (Manhasset, NY).

siRNA inhibition

The Ape1 siRNA sequence first detailed by Wang et al.\(^{33}\) and also employed by Courv-Privat et al.\(^{34}\) was used in this study. Double-stranded siRNA oligonucleotides (5’-GUCUG-GUAAGAGGAGUACC-3’, 5’-UACUCUGUACC-AGCCU-3’) were obtained from Ambion (Applied Biosystems/Ambion, Austin, TX) as was a non-specific siRNA control (Ambion cat# AM4613). siRNAs were transfected with Lipofectamine 2000, and after 48 hrs cells were irradiated at the doses indicated and subjected to clonogenic survival assay as described above (initial seeding densities in 100 mm dishes for U251/pCMV10 and U251/CMV-Ape1-5 were 250–12,000 and 125–6,000 respectively).

RESULTS

High basal level of Ape1 is associated with high levels of radioresistance

The U87 cell line is more radioresistant than the U251 line. As seen in Fig. 1, U87 showed significant cell death only at 500 cGy or higher doses of X-rays whereas U251 showed significant cell death even at 50–100 cGy. With a 1500 cGy dose, U251 cells had very few colony survivors (< 0.2%) whereas U87 had about 6% survivors. These results are in accordance with that reported by Yount et al.,\(^{35}\) Delmas et al.,\(^{36}\) and Eshleman et al.\(^{37}\) Since Ape1 activity has been shown to be significantly higher in human gliomas as compared to other cancers and normal cells, and since the association between Ape1 levels and radiation resistance varies in different cell types, we wanted to know

if these glioma cell lines differed in their Ape1 expression. The U87 cell line has higher Ape1 levels than the U251 line. As shown in Fig. 2A, in normal cycling cells, Ape1 protein

![Image](https://via.placeholder.com/150)
in U87 was 6.5 fold higher than in U251. For the Western blot, tubulin was used as a loading control, and purified, recombinant Ape1 (250 ng) was used as the positive control. Figure 2B shows a similar increase (about 5-to 6-fold) in the Ape1 endonuclease activity assayed in U87 as compared to U251, agreeing well with the Ape1 protein data. Ape1 endonuclease activity was assayed in these cells with pyruvate kinase activity as the second background control to normalize Ape1 activity between U87 and U251. The comparative data suggest that high Ape1 levels are associated with radioresistance. There may be other important differences between the two cell lines. To address this question, we sought to manipulate the amount of Ape1 in a controlled experimental system as follows.

Modulation of radioresistance in U251 by Ape1 overexpression

In order to define the specific role of Ape1 in radioresistance, the radiosensitive cell line U251 was engineered to overexpress Ape1. U251 cells were transfected with a pCMV-Ape1 plasmid and multiple Ape1 overexpressing clones were isolated. Ape1 protein expression was determined for each clone. As shown in Fig. 3A, clone 5 showed highest Ape1 protein expression followed by clones 1 and 6. The corresponding Ape1 endonuclease activity for the overexpressers is shown if Fig. 3B. Exposure of the three clones to 25–1500 cGy of X-rays demonstrated increased radioresistance (Fig. 3C), which correlated to increased Ape1 levels. A two component fit calculation was done for the survival curves in Fig. 3C between 500–1500 cGy. The increases of the surviving fraction of overexpressers relative to the pCMV10 control were: clone 6 = 3.5-fold; clone 1 = 4-fold; and clone 5 = 7.5-fold. There was a clear dose-dependent effect of increasing Ape1 overexpression and increasing radioresistance – even the 3.5-fold level of Ape1 overexpression yielded higher radioresistance than control transfected or non-transfected cells. The D37 (dose at which there is 37% survival) also increased with increasing Ape1 expression: clone 6 (3.5 – fold) = 170 cGy; clone 1 (9-fold) = 200 cGy; and clone 5 (24-fold) = 360 cGy.

Modulation of radioresistance by inhibition of Ape1 with lucanthone or CRT0044876

The converse of the above experiments, namely suppressing Ape1 and observing reduced radiation resistance (i.e., radiosensitization), would be an additional confirmation of the role of Ape1 in radioresistance. Such suppression might be attained by genetic (antisense, siRNA) or pharmacological (inhibitors) means. At least two inhibitors of Ape1 have been reported, CRT0044876 and lucanthone – the latter is of particular interest in this context due to its prior association as an adjunct to radiotherapy, including brain tumors. The most recent clinically relevant levels of lucanthone used as a radiosensitizer for treatment of glioblastomas with 3000 cGy whole brain irradiation (8 MeV) was reported to be 8–12 μM in patients’ serum (see, United States Patent #6391911). To determine an effective inhibitor concentration in the culture systems employed in this study, U87 and U251 cultures were subjected to doses of lucanthone ranging 5–100 μM for 2 h and assessed for their cell viability. Increasing the inhibitor concentration produced significant cellular toxicity, but that the lowest (5 μM) pretreatment resulted in 6% cell death in U251 and 13% in U87 (data not shown). Figure 4A shows that increasing the inhibitor con-
concentration produced significant cytotoxicity in Ape1 overexpressers as compared to cells expressing control plasmids. Of the two inhibitors used, lucanthone showed significantly higher cytotoxicity as compared to CRT0044876 and this higher lucanthone induced cytotoxicity was also seen in control plasmid expressing cells, which may be indicative of additional Ape1 independent effects of lucanthone as Ape1 inhibition has been shown to enhance cytotoxicity. In control conditions without irradiation, pretreatment with 5 μM lucanthone (the effective dose which showed cytotoxicity in Fig. 4. Decrease in radiation resistance by lucanthone and CRT0044876. (A) Cytotoxicity of Ape1 inhibitors assessed by clonogenic survival assay at the concentrations indicated. (B) Radiation sensitivity U87 and U251 pretreated with 5 μM lucanthone (left side) or 50 μM of CRT0044876 (right side) assessed by clonogenic survival assay. (C) Ape1 endonuclease activity of cell extracts from CMV control (pCMV10) and Ape1 overexpressing Clone 5 (Ape1-5) treated with 5 μM lucanthone, 50 μM of CRT0044876, or both for 2 hr.
our cell toxicity studies *in vitro* or 50 μM CRT0044876 concentration did not produce any measurable effect in either U87 or U251 clonogenic survival (Fig. 4B).

In all cases, lucanthone reduced radiation resistance as determined by clonogenic assay (Fig. 4B, left side figure). This reduction in radiation resistance was very significant with U87 as compared to U251, with it being more evident at higher doses (> 250 cGy or more). In contrast, the lower reduction in radiation response of U251 was independent of the dose. In all cases, as expected, U251 cells remained more radiosensitive than U87 cells. However, CRT0044876 did not show as significant a change in radiosensitivity as did lucanthone in U87 (Fig. 4B, right side figure), indicating that other DNA repair pathways are involved in addition to BER in the radiation response of these glioma lines. The specific effect of these inhibitors on Ape1 endonuclease activity was compared. Figure 4C shows the effect of both lucanthone and CRT0044876, alone and in combination, on the endonuclease activity in control transfected cells and Ape1 overexpressing Clone 5 cells. A significant inhibition by 5 μM lucanthone was seen, compared to a much lesser inhibition by 50 μM CRT0044876. In this glioma model system, these data indicate that the more potent inhibition of Ape1 activity is associated with a greater reduction of radioresistance.

**Modulation of radioresistance by siRNA inhibition of Ape1**

Inhibition of Ape1 by siRNA was done to confirm that knock down of Ape1 expression (Fig. 5A) and activity (Fig. 5B) had a corresponding effect on radiosensitivity (Fig. 5C). After 48 h, 50–70% inhibition of Ape1 in U87 and U251 revealed that suppressing Ape1 in these cells makes them more radiosensitive, much more than lucanthone as shown in Fig. 4.

**Transient G2/M arrest in irradiated cells of varying Ape1 expression.**

As irradiation predominantly affects the cell cycle and radiosensitivity depends on cell cycle position and cell cycle progression, along with the fact that U87 and U251 differed in their cell doubling times (U87 has a mean doubling time of ~53 hr, U251 has a mean doubling time of ~30 hr), the two cell lines were analyzed for their cell cycle stages before and after irradiation (Table 1). After 24 hr of subculture under normal conditions, we found that U87 was predominantly in the G1 stage (59%) with the remaining cells at 27% S and 13% G2/M. U87 cells showed a transient G2/M arrest (38% and 49%) between 6–12 hr post irradiation at 250 cGy, with no decrease in viability. In comparison, U251 cells under normal growth conditions were in 27% G1, 26% S and 47% G2/M stages. U251 cells showed a similar transient G2/M arrest (54% and 60%) as shown by U87 between 6-12 hr post irradiation at 250 cGy, but with an increase in sub G1/G0 (apoptotic) populations after 12 h.
Thus, we found that U251 cells which are more radioresistant than U87 cells have a higher proportion of cells to begin with in the G2/M stage, the most radioresistant part of the cell cycle, and have a greater accumulation in the G2/M stage following irradiation. Differences in cell cycle clearly play a role in the radiation tolerance of these cells – but only a partial role. While increased Ape1 was shown to increase radiation resistance (Figs. 1–5), it is possible that higher Ape1 drives cells away from the G2/M stage, rather than being a direct effect of the enzymatic function of Ape1. This is not the case. In the U251/Ape1 overexpression clones, despite variation in Ape1 levels, there was no alteration in the transient post-irradiation G2/M arrest or induced other cell cycle alterations without resulting in apoptosis (data not shown).

Ape1 protein expression is similar in both cell lines in G1 phase but in U87 shows a significantly higher Ape1 expression in S and G2/M phase

Since expression of Ape1 mRNA has been previously shown to be predominantly in the S phase and as our above results show that the two cell lines differed in their cell cycle stages, we wanted to determine if the cell cycle dependent expression of Ape1 affected the cell stage arrest with subsequent outcomes in terms of radiation responses in these two cell lines. As shown in Fig. 6, Ape1 protein expression in U87 is higher in S and G2/M (where both the cell lines arrested in > 95% in either S or G2/M phases). In contrast, Ape1 expression in U251 is predominantly in G1 and S phases. U87 showed about 2 fold and 3 fold increases in Ape1 protein in S and G2/M stages as compared to U251, but their Ape1 endonuclease activity levels were only higher in G2/M (~5 fold) but not in the S phase (Table 2). Moreover, the Ape1 endonuclease activities in G2/M phase was much higher than in S phase for U87 where as for U251 cell line it was lower, indicative of a differential regulation of Ape1 in these cell cycle stages between the two cell lines. We could not conclusively infer from the G1 data for Ape1 protein since both the cell lines could not be arrested in G1 significantly by serum starvation (only about 54% in U251 and 75% in U87).

### DISCUSSION

The possibility of a relationship between Ape1 activity and radioresistance has previously been noted, and there are multiple lines of evidence to support this notion. For instance, there are examples of high levels of Ape1 being associated with radioresistance. In radiosensitive *E. coli* mutant bacteria deficient in AP nuclease activity, transfec-
Glioma Radioresistance Through Ape1/Ref-1

In addition, there are examples of low levels of Ape1 associated with lowered radioresistance, e.g., increased radiosensitivity in lung carcinoma cells caused by reduction of Ape1 with antisense RNA, or radiosensitization of prostate tumor cell cultures or prostate tumor-bearing animals caused by reduction of basal Ape1 levels. The latter study further included experiments in which the isoflavone treatment of transfected cells overexpressing Ape1 caused radiosensitization due to very significant decreases of Ape1 levels. However, a relationship between Ape1 activity and radioresistance is not universal. There are counter-examples when high levels of Ape1 have no effect on radioresistance. Also, a survey of several glioma cell lines (HRB84T, KMG4, T98G, HRB17T, A172, U251) revealed that the mRNA expression levels generally correlated with endonuclease activity levels and radioresistance, except for the A172 line, which did not. And there are instances where lowering Ape1 does not affect radiosensitivity.

The variety of results is partly a reflection of the variety of experimental models, thus pursuing the issue of Ape1 and radiosensitivity requires re-examination in appropriate models. Glioblastomas continue to be among the solid tumors most resistant to therapy, and when glioma cell lines are evaluated in monolayer culture, their chemo- and radiosensitivities are sometimes no different from cell lines originating from other histologies that typically respond to treatment. Moreover, cell cultures in vitro can behave differently than xenografts, and among in vivo models, orthotopic tumors are generally regarded to be superior models compared to non-orthotopic tumors in more closely mimicking the naturally occurring cancer. In the specific case of the cell lines used in this study, U87 and U251, it is known that they exhibit very different gene expression profiles when studied (i) as in vitro cell cultures, (ii) as subcutaneous xenograft tumors in SCID mice, and (iii) as orthotopic, intracranial tumors in SCID mice. An ideal way to pursue Ape1 in glioma radioresistance is, perhaps, exemplified in the recent paper by Raffoul and colleagues. They studied Ape1 regulation simultaneously in a prostate cell culture system and in an orthotopic prostate xenograft tumor model and their study provides convincing evidence about the role of Ape1 and radiosensitization.

In the review by Evans et al., different studies are discussed in which Ape1 is raised or lowered by overexpression or antisense inhibition. Since then, additional relevant studies have added to the body of literature on this subject. The expectation is that healthy normal cells could be afforded added protection from the toxic and mutagenic effects of DNA damaging agents, including ionizing radiation. This appears to be generally the case. What is not clear is whether the enhanced sensitivity of cells to these agents results from the DNA repair function (“Ape1”) or its non-DNA repair function (“Ref-1”). Our studies, on the other hand, indicate that Ape1 plays a significant role in the radiation sensitivity of two different glioma cell lines and that this is due to measurable and significant differences in the AP endonuclease activity. In our Ape1 overexpression studies in the radiosensitive cell line U251, we clearly demonstrate the dependence of radioresistance on Ape1 levels– the highest Ape1-expressing

### Table 2

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**Ape1 endonuclease activity (fmoles/min/mg)**

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**Pyruvate Kinase activity (PKU/mg)**

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<td>S</td>
<td>202*</td>
<td>189*</td>
<td>960*</td>
<td>110*</td>
</tr>
<tr>
<td>G2/M</td>
<td>202*</td>
<td>189*</td>
<td>960*</td>
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*10^6

Fig. 6. Ape1 protein levels during S and G2/M phases of cell cycle. U87 and U251 were arrested in G1 (by serum starvation for 96 h), S (by 2 mM Thymidine for 48 hr) and G2/M (by 40 ng/ml of Nocodazole for 24 hr), harvested and assessed for Ape1 protein by Western blot. As can be seen from this figure both the cell lines could not be arrested in G1 significantly by serum starvation. Total protein loaded per lane was normalized for tubulin protein.
clones exhibited the highest radioresistance, and the lowest Ape1-overexpressing clones exhibited the lowest radioresistance (Fig. 3). In this study we did not measure the redox activity of Ape1, as Kelley and colleagues29 had previously shown that lucanthone affects only the repair activity of Ape1 without affecting its redox activity or exonuclease activity for mis-matched nucleotides. And though we cannot exclude the role of other BER enzymes like XRCC1 or other DNA repair enzymes such as Nth, PARP-1 in the outcomes of Ape1 overexpression (especially since Ape1 is shown to suppress SSB induced PARP-1 activation,31) and Ape1 and PARP-1 compete for the same intermediate in BER32) and recombination proteins (Ku70/80 heterodimer), we can infer that the changes in radiation sensitivity we observed throughout the study are due to the AP endonuclease activity of Ape1 based on the specific inhibition by lucanthone (Fig. 2).

This work and the related studies discussed above suggest that Ape1 may be a useful target to modulate tissue responses to radiation therapy, and lucanthone is an example of an Ape1-targeting agent. It has been investigated as an adjunct to radiotherapy in human cancer patients with pulmonary metastases and oropharyngeal tumors,28) cervical cancer29) and brain metastases.30) The closely related hycanthone, whose mechanistic action is the same as lucanthone,39) was also evaluated as a radiotherapy adjuvant in a Phase III clinical trial for pancreatic cancer.27) The use of these compounds did in fact act as tumor radiosensitizers, but in most cases the radiosensitization of normal tissue provoked deleterious side effects. Because lucanthone efficiently crosses the blood brain barrier,30) it remains in the investigation pipeline as Thiianthenone for brain tumor radiotherapy. Lucanthone (CAS 479-50-5) and hycanthone (CAS 3105-97-3) are thioxanthenes (a.k.a. thioxanthones), which were known as DNA-intercalating agents and were originally developed for human use as anti-parasitic drugs. A number of DNA-intercalating agents are used in cancer chemotherapy (e.g., daunomycin, mitoxantrone, etc.), but that activity is not the sole mode of action – some of these drugs also interfere with the breakage-ligation reaction catalyzed by DNA topoisomerase II. Lucanthone and hycanthone are also included in this group, and it was as topoisomerase inhibitors that they were considered to hold promise as chemotherapy and radiotherapy sensitizers. However, lucanthone and hycanthone additionally interfere with AP endonuclease, independent of DNA intercalation,25,26) and we have begun to characterize the physical interaction of lucanthone and Ape1. But its mode of action is more complex than the earliest reports, which should now be regarded to include direct Ape1 inhibition. CRT0044876 was used as positive control for Ape1 inhibition; however it was not as good a radiosensitizer as lucanthone, which we attribute to its lower Ape1 endonuclease inhibitory activity.

Finally, no consideration of altered radiation sensitivity can take place unless cell cycle effects are evaluated since a variety of findings suggest Ape1 modulation with the cell cycle. Parlanti et al.33) showed that Ape1, along with other components of BER like uracil DNA glycosylase (UNG2), XRCC1, POLβ, DNA PK, replicative POLα, δ and ε and DNA ligase 1, was physically associated with cell cycle regulatory proteins cyclin A and DNA replication protein MCM7. Ape1 regulation in cell cycle is re-enforced by their finding of physical association between major S-phase protein like cyclin A and Ape1. Fung et al.24) have shown that Ape1 mRNA expression seems to be most elevated in mid-S phase and the increased Ape1 mRNA was the result of transcriptional activation rather than increased mRNA stability. Cdc25B expression is regulated by redox signaling35) and Ape1 protein is also considered to have redox regulatory function for several transcription factors and is known to regulate cell cycle status in hematopoietic progenitors and G1/S transition in embryonic bodies (EB) cells,55) so it is possible that Ape1 regulates Cdc25B by its redox function and thereby affects the G2/M transition of these glioma cells. p53 has a major role in G1 cell cycle delay,30) which may be important for Ape1 activity since p53 and Ape1 have been known to bind each other, albeit without any apparent changes in either p53 function/Ape1 repair activity.57) As shown by Zaky et al.58) wild type p53 negatively regulates Ape1 expression, thus it might be possible to see its negative regulation on Ape1 expression in these glioma cells, if M phase synchronized cells are employed in the manner of Yount et al. for irradiated U87 cells.59)

Addressing all of these issues is complex, but our data allows general, preliminary inferences. In light of these previous findings, our data which show lower Ape1 protein/endonuclease activity in G2/M of U251 as compared to U87 probably correlate to lower cell survival of this cell line as most of its cells are in the radiosensitive G2/M phase.60) It is possible that Ape1 protein may be expressed in both the cell lines but was inhibited in U251 G2/M phase from being as active as in U87, which has 3 fold higher activity. Or, Ape1 synthesized during the S phase is still present in G2/M as the mRNA may persist beyond S phase and still be translated, with a differential regulation possibly controlled by other cell cycle proteins. Since a significant amount of U251 cells reside in G2/M phase (46%), due to less active Ape1 in this phase these cells may be becoming more radiosensitive than U87 which has a very active Ape1 in this phase; though U87 cells predominantly reside in G1 phase which is a more radiosensitive cell phase. While these possibilities are of interest, they would limit the conclusions we could draw if the data were limited to correlations of Ape1 activity between two intrinsically different cell lines, however, the Ape1 overexpression experiments (Fig. 3) and siRNA inhibition experiments (Fig. 5) more definitively link Ape1 activity to radioresistance. Contrary to our expectations, Ape1 overexpression did not remove the transient 6–12 h
G2/M arrest shown by parental U251 cells after exposure to 250 cGy of X-rays, but it did inhibit the 2–3% apoptosis shown by parental U251, thus underscoring the need for further studies with cell cycle stages arrest of these parental and Ape1 overexpresser U251 glioma cell lines in G1, S and G2/M, which may confirm the cell cycle stage dependency of Ape1 expression as one of the ways Ape1 expression correlates with radioresistance. Our analysis suggests that while cell cycle variation of Ape1 expression may influence radiation induced cell death, Ape1’s role as a root cause of radiation tolerance is largely unaltered by complexities of cell cycle.

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