Modulating Roles of Amiloride in Irradiation-Induced Antiproliferative Effects in Glioblastoma Multiforme Cells Involving Akt Phosphorylation and the Alternative Splicing of Apoptotic Genes

Jen-Yang Tang,^{1–3} Hsueh-Wei Chang,^{3,4} and Jan-Gowth Chang^{5–7}

Apoptosis is a key mechanism for enhanced cellular radiosensitivity in radiation therapy. Studies suggest that Akt signaling may play a role in apoptosis and radioresistance. This study evaluates the possible modulating role of amiloride, an antihypertensive agent with a modulating effect to alternative splicing for regulating apoptosis, in the antiproliferative effects induced by ionizing radiation (IR) in glioblastoma multiforme (GBM) 8401 cells. Analysis of cell viability showed that amiloride treatment significantly inhibited cell proliferation in irradiated GBM8401 cells ($p < 0.05$) in a time-dependent manner, especially in cells treated with amiloride with IR post-treatment. In comparison with GBM8401 cells treated with amiloride alone, with GBM8401 cells treated with IR alone, and with human embryonic lung fibroblast control cells (HEL 299), GBM8401 cells treated with IR combined with amiloride showed increased overexpression of phosphorylated Akt, regardless of whether IR treatment was performed before or after amiloride administration. The alternative splicing pattern of apoptotic protease-activating factor-1 (*APAF1*) in cells treated with amiloride alone, IR alone, and combined amiloride-IR treatments showed more consistent cell proliferation compared to that in other apoptosis-related genes such as baculoviral IAP repeat containing 5 (*BIRC5*), *Bcl-X*, and homeodomain interacting protein kinase-3 (*HIPK3*). In GBM8401 cells treated with amiloride with IR post-treatment, the ratio of prosurvival (-XL,-LC) to proapoptotic (-LN,-S) splice variants of *APAF1* was lower than that seen in cells treated with amiloride with IR pretreatment, suggesting that proapoptotic splice variants of *APAF1* (*APAF1*-LN,-S) were higher in the glioblastoma cells treated with amiloride with IR post-treatment, as compared to glioblastoma cells and fibroblast control cells that had received other treatments. Together, these results suggest that amiloride modulates cell radiosensitivity involving the Akt phosphorylation and the alternative splicing of *APAF1*, especially for the cells treated with amiloride with IR post-treatment. Therefore, amiloride may improve the effectiveness of radiation therapy for GBMs.

Introduction

IRRADIATION CAUSES DNA damage and induces cell death via apoptosis, mitotic catastrophe, autophagy, or senescence (Dunne *et al.*, 2003; Ishikawa *et al.*, 2006; Chen *et al.*, 2012; Salem *et al.*, 2012). However, since cells may survive DNA damage by activating survival signal pathways (Cataldi *et al.*, 2009), cellular radiosensitivity, an important factor in the tumor response to radiotherapy, may be affected by the balance between cell death and survival.

The phosphoinositide 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog 1 (Akt) signaling pathway is

essential for regulating cell proliferation and is often deregulated in cancers (Willems *et al.*, 2012). The PI3K/Akt signaling pathway is also a therapeutic target in grade IV brain tumors (Holand *et al.*, 2011). Upregulated Akt activity and levels are reportedly associated with radioresistance (Shimura, 2011), and Akt signaling may have an important role in apoptosis (Liang and Slingerland, 2003; Lu *et al.*, 2008; Gu *et al.*, 2011; Xu *et al.*, 2012) and radioresistance (Shimura, 2011).

Apoptosis is a key mechanism for achieving an effective therapeutic response to radiation therapy (Salem *et al.*, 2012). This programmed cell death is activated by distinct

¹Department of Radiation Oncology, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan.

²Department of Radiation Oncology and ³Cancer Center, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan.

⁴Department of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung, Taiwan.

⁵Epigenome Research Center, China Medical University Hospital, Taichung, Taiwan.

⁶Graduate Institute of Integrated Medicine, College of Chinese Medicine, China Medical University, Taichung, Taiwan.

⁷School of Medicine, China Medical University Hospital, Taichung, Taiwan.

cellular stresses, such as growth factor withdrawal and DNA damage (Letai, 2006). Several genes, such as apoptotic protease-activating factor-1 (*APAF1*), survivin (baculoviral IAP repeat containing 5 [*BIRC5*]), and homeodomain interacting protein kinase-3 (*HIPK3*), are known to have roles in cellular apoptotic processes. For example, because *APAF1* homo-oligomerizes into a caspase-activating complex, it plays an essential role in apoptotic signaling. By recruiting and activating caspase-9, the complex forms apoptosome, which results in a proteolytic cascade and the activation of caspase-3 and caspase-7 (Bratton and Salvesen, 2010). The *BIRC5* directly inhibits apoptosis by binding to caspases (Filippi-Chiela *et al.*, 2011). The *HIPK3* negatively regulates Fas-mediated apoptosis, the dysfunction of which is associated with several diseases such as autoimmune disease and cancer (Stupp *et al.*, 2005). Since modulated expression of these apoptotic genes may affect apoptosis, these modulating effects may alter sensitivity to radiation therapy.

Damage to DNA can induce apoptosis, and several apoptotic genes may be modified via alternative splicing (Munoz *et al.*, 2009). Alternative splicing is an important post-transcriptional modification that generates diverse RNA isoforms and functions (Tiziano *et al.*, 2010; Wu *et al.*, 2012). Since many alternative splicing variants of apoptotic factors have antagonistic functions (Schwerk and Schulze-Osthoff, 2005), studies suggest that the effectiveness of cancer therapies may be improved by manipulating alternative splicing genes to increase radiosensitivity. Amiloride is a potassium-sparing diuretic used to treat hypertension (Bull and Laragh, 1968). Recently, Chang *et al.* (2011b) discovered that amiloride plays a novel biological role in modulating alternative splicing and in inducing apoptosis in leukemic cells. Amiloride also has a transcriptome-wide effect on the alternative splicing of RNA transcripts, particularly in apoptotic genes (Chang *et al.*, 2011b).

Radiotherapy is the standard therapy for high-grade glioblastoma such as glioblastoma multiforme (GBM) (Yasui and Owens, 2012). However, its efficacy is limited by the radioresistance that is generated during the course of irradiation (Bao *et al.*, 2006; Fedrigo *et al.*, 2011; Filippi-Chiela *et al.*, 2011). Therefore, this study investigated the possible modulating effect of amiloride on irradiation-induced anti-proliferation of human brain GBM8401 cells and evaluated its effects on Akt signaling and on alternative splicing in four apoptotic genes.

Materials and Methods

Cell culture and ionizing radiation exposure

Human GBM8401 cells and human fibroblast HEL 299 cells obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan) were grown in the DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, and 1% penicillin/streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO₂. Amiloride (Sigma Chemical) treatment was performed as previously described (Chang *et al.*, 2011a). At specified times, cells were irradiated with a single dose of 5 Gy at room temperature by a Varian 2100 C/D linear accelerator (Varian Medical System) at a dose rate of 300 MU/min (3 Gy/min) and at a source-to-target distance of 100 cm.

Cell viability inhibition assay

The trypan blue dye exclusion method (Chiu *et al.*, 2009; Yen *et al.*, 2010) was used to measure the viability of cells treated with 0.2 mM amiloride and/or 5 Gy ionizing radiation (IR) for the time intervals of 24, 48, and 72 h. Exponentially grown cells (1×10^4) were plated in 96-well plates until 90% confluence was achieved. The cells were then treated with 0.2% trypan blue and counted with a hemocytometer (Sigma-Aldrich). Each treatment was performed in triplicate.

Western blot analysis

Cells treated with 0.2 mM amiloride and/or 5 Gy IR were harvested and denatured by mixing with a lysis buffer at 4°C for 20 min. After centrifuging the lysates, protein concentrations were determined with a BSA protein assay kit (Pierce). Equal amounts of proteins were loaded onto sodium dodecylsulfate–polyacrylamide (10%) gels for electrophoresis and transferred to polyvinylidene difluoride membranes. Each membrane was then blocked with 5% nonfat dry milk in a TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) for 1 h and washed with the same buffer. After incubation with the desired primary antibodies, the cells were washed and treated with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare Bio-Sciences) according to the manufacturer's instructions. The signal intensities were measured using LabWorks software (UVP BioImaging Systems).

RNA extraction and reverse transcription–polymerase chain reaction for alternative splicing analysis

After treatment with 0.2 mM amiloride and/or 5 Gy IR, mRNA was extracted from the cells using a TurboCapture 8 mRNA kit (Qiagen) according to the manufacturer's instructions. Reverse transcription (RT) of the mRNA was performed using a mixture of oligo(dT), random primers, and Moloney murine leukemia virus reverse transcriptase (Promega). Table 1 shows the specific primer pairs used for polymerase chain reaction (PCR). In these genes, the ratios of pro-survival splice variants to pro-apoptotic splice variants were calculated according to their relative gel densities measured using the UVP BioImaging Systems with LabWorks software.

Statistical analysis

All results are expressed as mean $\pm$ SD. The Student's *t*-test was performed to determine significant differences between experimental groups. A *p*-value of <0.05 was considered statistically significant.

Results

Effect of amiloride on irradiation-induced cell death

Figure 1 shows that, compared to cells treated with amiloride alone or with IR alone, the cell death effect was enhanced in cells treated with amiloride with IR pre- and post-treatments. Additionally, cell death was significantly higher in cells treated with amiloride with IR post-treatment as compared to cells treated with amiloride with IR pre-treatment ($p < 0.05$). All these treatments displayed anti-proliferation in a time-dependent manner.

TABLE 1. PRIMERS USED FOR REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION ANALYSIS OF FOUR ALTERNATIVE SPLICING GENES

Genes	Forward primers	Reverse primers	Tm ^a	Size (bp) ^b
APAF1	CAGCTGATGGAACCTTAAAGC	GTCTGGTCATCAGAAGATGTC	62.0	430, 301
BIRC5	CACCGCATCTCTACATTCAA	CACTTCTCCGCAGTTTCCT	60.0	345, 227
Bcl-X	GAGGCAGGCGACGAGTTTGAA	TGGAGGGTAGAGTGGATGGT	56.0	460, 271
HIPK3	AGCCTGCCACTACCAAGAAA	CAGCAATTTCTTGCCTCTCC	58.0	244, 181

^aAnnealing temperature for polymerase chain reaction.

^bAlternative splicing size.

APAF1, apoptotic protease-activating factor-1; BIRC5, baculoviral IAP repeat containing 5; HIPK3, homeodomain interacting protein kinase-3.

Effect of IR and amiloride on phosphorylation of Akt protein

Since the PI3K/Akt pathway is essential for regulating cell proliferation and survival, this study first evaluated Akt and phosphorylated Akt (p-Ser-Akt) levels in GBM8401 cells treated with IR alone, amiloride alone, and amiloride of IR pre- and post-treatments. None of the treatments significantly affected Akt levels in the cells (Fig. 2). However, phosphorylated Akt (p-Ser-Akt) levels were significantly higher in cells treated with amiloride with IR post-treatment (after; lanes 4–8) and in cells treated with amiloride with IR pretreatment (before; lanes 9–14) compared to untreated control cells, cells treated with amiloride alone, and cells treated with IR alone (lanes 1–3, respectively). These data

suggest that the amiloride/IR combined treatments, that is, both IR (after) and IR (before), activated Akt phosphorylation in the GBM8401 glioblastoma cells. In contrast, in human embryonic lung fibroblast control cells (HEL 299) treated with amiloride and/or IR (Fig. 2B), phosphorylated Akt levels were only half those observed in glioblastoma cells (Fig. 2A). Notably, IR reportedly induces Akt phosphorylation in human hepatocellular carcinoma HepG2 cells and in cervical cancer HeLa cells (Shimura, 2011).

Effect of amiloride and IR on alternative splicing of apoptotic genes

In amiloride-treated cells for IR post- and pretreatments (Fig. 3A, B, respectively), proapoptotic splice variants of APAF1 (APAF1-LN,S) increased concomitantly with a decrease in the prosurvival splice variants APAF-XL,LC. The ratio of prosurvival splice variants of APAF1 to proapoptotic splice variants of APAF1 was lower (Fig. 3C) than that found in controls and was positively correlated with cell survival (Fig. 1). In APAF1, treatment with IR alone did not substantially change the -XL,LC/-LN,S ratio (Fig. 3C) and nor did it dramatically increase cell death as compared to the combined treatments with amiloride and IR (Fig. 1).

Cells treated with amiloride with IR post-treatment also showed a lower ratio of -XL,LC/-LN,S as compared to cells treated with amiloride with IR pretreatment. Although the difference did not reach a statistical significance ($p=0.06$), cells treated with amiloride with IR post-treatment had more proapoptotic splice variants (APAF1-LN,S) of APAF1 and fewer prosurvival splice variants (APAF-XL,LC) as compared to those treated with amiloride with IR pretreatment. These observations imply that amiloride modulates radiosensitivity by regulating the alternative splicing of the APAF1 gene. A similar pattern of changes was noted in proapoptotic and prosurvival splice variants of BIRC5, but the differences were not statistically significant.

In contrast, the ratios of prosurvival splice variants of Bcl-X and HIPK3 [Bcl-X_L and HIPK3 U(+)] to their respective proapoptotic variants [Bcl-X_S and HIPK3 U(-); Fig. 3C] did not correlate with changes in cell survival resulting from amiloride treatment with IR post- and pretreatments (Fig. 1).

Effects of amiloride combined with pre- or post-treatment with IR on alternative splicing of APAF1 at different time points

The effects of amiloride treatment performed before and after IR were further confirmed in terms of its modulation

FIG. 1. Viability of glioblastoma multiforme (GBM) 8401 cells treated with amiloride with ionizing radiation (IR) pre- and post-treatments. Viabilities of GBM8401 cells treated with IR alone, with 0.2 mM amiloride alone, with amiloride with IR pretreatment (IR→amiloride), and with amiloride with IR post-treatment (amiloride→IR) for varying incubation times. In cells treated with amiloride alone or with IR alone, cell viabilities were measured over three time intervals. In the IR→amiloride cells, irradiation with 5 Gy was immediately followed by incubation with amiloride for three time intervals. The amiloride→IR cells were treated with amiloride for 24 h, washed, irradiated with 5 Gy, and then incubated in an amiloride-free medium for three time intervals.

FIG. 2. Expressions of the p-Akt protein were measured at different incubation times in GBM8401 cells treated with amiloride with IR post-treatment (amiloride→IR) and in GBM8401 cells treated with amiloride with IR pretreatment (IR→amiloride). (A) The amiloride→IR cells and IR→amiloride cells were incubated for the indicated times. (B) Similar experiments were performed in human fibroblast HEL 299 cells. The amiloride→IR cells were treated with 0.2 mM amiloride for 24 h, irradiated with IR 5 Gy, and then incubated in an amiloride-free medium for varying durations. The IR→amiloride cells were irradiated with IR 5 Gy and immediately incubated with amiloride for varying durations. The control cells were not treated with either amiloride or IR. The p-Ser-Akt/Akt ratios for protein levels in the treated cells were calculated in reference to the control cells. Lanes indicate the conditions for control, amiloride with IR pre- or post-treatments with different incubation times.

of *APAF1* splicing in glioblastoma GBM8401 cells (Fig. 4A) and in human embryonic lung fibroblast control HEL 299 cells following different incubation times. In amiloride-treated GBM8401 cells incubated for 0–24 h (Fig. 4A, lanes 3–8), IR post-treatment increased proapoptotic splice variants of *APAF1* (*APAF1*-LN, -S), but decreased prosurvival splice variants of *APAF1* (*APAF1*-XL, -LC). Compared to amiloride-treated GBM8401 cells with IR pretreatment, those amiloride-treated GBM8401 cells with IR post-treatment had a lower ratio of prosurvival *APAF1* splicing

FIG. 3. Effects of combined treatment with amiloride and IR on the alternative splicing of various apoptotic genes in GBM8401 cells. (A) The results of reverse transcription–polymerase chain reaction (RT-PCR) analysis showed that alternatively spliced isoforms of PCR products in four apoptotic genes (A) were amplified by the individual variant-specific primers shown in Table 1. (B) The RT-PCR products were resolved by agarose gels. (C) In densitometric analyses of the agarose gels, relative intensities of individual spliced variants were normalized to those of the untreated controls. Cells treated with amiloride (Ami) alone were incubated for 24 h in 0.2 mM amiloride, washed, and then incubated in an amiloride-free medium for another 24 h. Cells treated with IR alone were irradiated with 5 Gy and incubated in an amiloride-free medium for 24 h. In cells treated with amiloride with IR post-treatment (amiloride→IR), 24-h treatment with amiloride was followed by washing, irradiation with 5 Gy, and a 24-h incubation in an amiloride-free medium. In cells treated with amiloride with IR pre-treatment (IR→amiloride), irradiation with 5 Gy was immediately followed by a 24-h incubation with amiloride. *Significant difference between cells treated with amiloride with IR pre- and post-treatments.

FIG. 4. Expression of apoptotic protease-activating factor-1 (*APAF1*) splicing variants after varying incubation times for GBM8401 cells treated with amiloride with IR post-treatment (amiloride→IR) or with amiloride with IR pretreatment (IR→amiloride). **(A)** The amiloride→IR cells were treated with 0.2mM amiloride for 24h, irradiated with IR 5 Gy, and then incubated in an amiloride-free medium for varying durations. The IR→amiloride cells were irradiated with IR 5 Gy and then immediately incubated with amiloride for varying durations. **(B)** Similar experiments were performed in human fibroblast HEL 299 cells. Lanes indicate the conditions for control, amiloride with IR pre- or post-treatments with different incubation times.

variants to proapoptotic *APAF1* splice variants, indicating an increase in apoptotic cell death. In contrast, in amiloride-treated cells with IR pretreatment (Fig. 4A, lanes 9–14), the prosurvival splice variants of *APAF1* (*APAF1*-XL, -LC) and proapoptotic splice variants of *APAF1* (*APAF1*-LN, -S) did not substantially differ for incubation lasting from 0 to 4 h.

In human embryonic lung fibroblast control cells (HEL 299), amiloride combined with either IR pre- or post-treatment did not substantially change the ratios of *APAF1* splicing variants (Fig. 4B, lanes 1–14). Accordingly, the different responses of tumor and embryonic fibroblast cells to the combined treatment with amiloride and IR may reflect differences in expression in *APAF1* splicing variants.

Discussion

Recently, combined treatments with drugs, such as inhibitors of heat-shock protein 90 and cyclooxygenase-2, have been found to significantly enhance the therapeutic efficacy of IR (Kim and Pyo, 2012). Similarly, this study showed that amiloride treatment had an antiproliferative effect and increased radiosensitivity in irradiated GBM8401 glioblastoma cells. Earlier studies have shown that the antiproliferative effects of amiloride in several cancer cell types may result from apoptosis (Park *et al.*, 2009; Chang *et al.*, 2011a, 2011b). In contrast, other agents, for example, lactate and gemcitabine, are reported to, respectively, increase radioresistance (Hirschhaeuser *et al.*, 2011) and decrease radiosensitivity (Salem *et al.*, 2012). The current study of GBM8401 glioblastoma cells showed a larger antiproliferative effect in amiloride-treated cells with IR post-treatment compared to amiloride-treated cells with IR pretreatment. This finding supports the notion that the order of treatment with different

medications can substantially affect the outcomes of anti-cancer therapy.

Similarly, clinical studies show that the hypoxia-activated prodrug TH-302 induces cytotoxic injury in tumors when combined with chemotherapeutic drugs (e.g., docetaxel, cisplatin, pemetrexed, irinotecan, doxorubicin, gemcitabine, or temozolomide). Interestingly, the efficacy of TH-302 is improved when administered before rather than after treatment with one of the above chemotherapeutic agents. Accordingly, when TH-302 is used in a combination with other conventional antineoplastic agents, the efficacy of TH-302 is affected by the order in which it is administered (Liu *et al.*, 2012).

Irradiation has been reported to induce alternative splicing. For example, low-dose ultraviolet (UV) irradiation is known to regulate alternative splicing of the apoptotic gene *Bcl-X* in Hep3B cells (Munoz *et al.*, 2009). Exposure to UV decreases the ratio of prosurvival/proapoptotic isoforms of *Bcl-X*. The current study similarly found that IR induced alternative splicing. In amiloride-treated GBM8401 glioblastoma cells, IR post-treatment activated Akt signaling and concurrently decreased the prosurvival splice variants of *APAF1* (*APAF1*-XL, -LC). Moreover, the *APAF1*-XL, -LC variants are prosurvival splicing variants, and the *APAF1*-LN, -S variants are known to have proapoptotic functions during apoptosis (Benedict *et al.*, 2000). In GBM8401 glioblastoma cells treated with both amiloride and IR (IR pre- or post-treatment), prosurvival splice variants of *APAF1* were decreased, but those of *Bcl-X* and *HIPK3* were not. These results suggest that the discrepancy may result from different irradiation sources/dosages and cell types. Alternatively, the regulation of the splicing of *Bcl-X* and *HIPK3* may not be involved with Akt. For example, in our previous study (Chang *et al.*, 2011b), we

found that Akt inactivation was not responsible for the splicing of *Bcl-X* and *HIPK3* in terms of validation by PI3K inhibitor treatment in leukemic cells. In the current study, the different apoptotic radiosensitivities obtained by different orders of treatment correlated well with changes in the proapoptotic and prosurvival splicing variants of *APAF1*. In addition, ratios of *APAF1* prosurvival/proapoptotic isoforms do not differ much between treatment with amiloride alone and amiloride followed by irradiation. The role of *APAF1* splicing in the amiloride alone and the combined treatment of amiloride and irradiation needs to be further investigated.

In sum, this study found that amiloride treatment before or after IR decreased the proliferation of GBM8401 glioblastoma cells, especially in amiloride-treated cells with IR post-treatment. Combining amiloride treatment with IR has good potential for improving radiosensitivity and the efficacy of radiotherapy for human brain glioblastoma.

Acknowledgments

This study was financially supported by grants from the Kaohsiung Medical University Hospital Research Foundation (KMUH96-6G72), the Kaohsiung Medical University Research Foundation (KMUER001), the Department of Health, Executive Yuan, Republic of China (Taiwan; DOH102-TD-C-111-002), NSYSU-KMU Joint Research Project (#NSYSUKMU 102-034), and the National Science Council, Taiwan (NSC100-2321-B039-007 and NSC101-2320-B-037-049).

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Hsueh-Wei Chang, PhD

Department of Biomedical Science and Environmental Biology

Kaohsiung Medical University

100, Shichuan 1st Road

Sanming District

Kaohsiung 80708

Taiwan

E-mail: changhw@kmu.edu.tw

Jan-Gowth Chang, MD

Graduate Institute of Integrated Medicine

College of Chinese Medicine

China Medical University

Taichung 40402

Taiwan

E-mail: d6781@mail.cmuh.org.tw

Received for publication February 4, 2013; received in revised form May 28, 2013; accepted June 3, 2013.