Plasma-Activated Medium Selectively Kills Glioblastoma Brain Tumor Cells by Down-Regulating a Survival Signaling Molecule, AKT Kinase

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ABSTRACT: Glioblastoma brain tumor cells and normal astrocytes were treated with plasma-activated medium (PAM). Cell proliferation assays showed that glioblastoma cells were selectively killed by PAM. PAM induced morphological changes consistent with apoptosis in glioblastoma cells and the cells decreased in size. We confirmed that those cells induced apoptosis using an apoptotic molecular marker, cleaved Caspase3/7. To elucidate the molecular mechanisms of PAM-mediated apoptosis in glioblastoma cells, we investigated the effects of survival signal transduction pathways. We found that PAM downregulated the expression of AKT kinase, a marker molecule in a survival signal transduction pathway. These results suggest that PAM may be a promising tool for therapy of glioblastoma brain tumors by downregulating the survival signals in cancers.

KEY WORDS: Plasma-activated medium, glioblastoma, AKT kinase

I. INTRODUCTION

Three major cancer therapies are currently in use: surgery, radiation, and chemotherapy. Recently, medical applications using non-equilibrium atmospheric pressure plasma (NEAPP) have attracted attention in cancer therapy as an innovative technology.1–5 Some pioneering work has shown that NEAPP exerts anti-tumor effects on lung carcinoma6 and induces apoptosis in melanoma skin cancer cells.7 In addition, recent studies have shown that disruption in extracellular matrix molecules by plasma8,9 and ataxia-telangiectasia mutated (ATM)-independent and ATM and Rad3-related (ATR)-dependent phosphorylation of H2AX by plasma10 have anti-tumor effects on cancer cells. Thus, elucidating the molecular mechanisms responsible for the anti-tumor effects of NEAPP is an important frontier in plasma-based cancer therapy. NEAPP with ultra-high electron density (approximately \(2 \times 10^{16} \text{ cm}^{-3}\)) has been developed11,12 it has...
been previously applied to inactivate *Penicillium digitatum* spores, and it has been used as therapy for ovarian cancers. The fact that plasma affects cancer cells not only directly but also indirectly by changing the biological environment around the cells as begun to draw the attention of researchers. Plasma-activated solutions may have anti-tumor effects on cancer cells; thus, studies of plasma-activated solutions may lead to medical applications.

Glioblastomas are the most common malignant brain tumors in adults, and these tumors generally recur within a year, regardless of the initial response to treatments. It is often difficult to delineate tumor boundaries of glioblastomas. Thus, innovative cancer therapeutic strategies are needed to selectively kill glioblastomas. Vandamme et al. have shown that plasma reduces the size of tumors in animals with glioblastomas. However, further studies are needed to confirm and develop medical applications. For example, selectivity may be required, and plasma-activated solutions could be powerful tools in therapy for cancer cells that are dispersed around normal tissues, as with meningeal dissemination. The molecular mechanisms of the anti-tumor effects of plasma on glioblastomas must be determined to achieve these ultimate treatment goals.

Activation of the phosphatidylinositol 3-kinase/phosphatase and tensin homologue (PI3K/PTEN)-AKT pathway plays a pivotal role in the development of glioblastoma. The two main regulators in this pathway are PI3K and PTEN. *PTEN* is a tumor suppressor gene that encodes a phosphatase that counteracts the effect of PI3K. A central point in the signaling pathway is controlled by the serine/threonine kinase AKT. Most protein kinases in human are serine/threonine kinases, and many of them are important regulators of survival and growth. However, they have not been extensively studied in cancer therapy, in contrast to tyrosine kinases, which are also important regulators of survival and growth. AKT is activated by PI3K and negatively regulated by PTEN. In many human glioblastomas, gain-of-function by *PI3K* mutations or loss-of-function by *PTEN* mutations are observed, and AKT is constitutively activated.

In this study, we found that plasma-activated medium (PAM) killed not astrocyte normal human brain cells, but glioblastoma human brain tumor cells through induction of apoptosis. Our goal is to understand the intracellular molecular mechanisms by which PAM induces apoptosis in glioblastomas, and we found that PAM downregulated the expression of AKT kinase in glioblastomas.

### II. MATERIALS AND METHODS

#### A. Experimental Setup for Producing Plasma-Activated Medium (PAM)

A diagram depicting the experimental setup is shown in Figure 1. Three milliliters of fresh medium in a 6-well plate was treated with plasma for several minutes, and 200 μL of this plasma-treated medium was used to replace the medium on the cells in the 96-well plate. NEAPP with ultra-high electron density (approximately $2 \times 10^{16}$ cm$^{-3}$) was produced with an estimated O density of approximately $4 \times 10^{15}$ cm$^{-3}$, as described...
FIGURE 1: Schematic for producing PAM. Cells were plated in 200 μL medium in a 96-well plate. On the following day, 3 mL fresh medium in a 6-well plate was treated with plasma, Ar gas, or was not treated for several minutes (L = 13 mm, 2.0 slm), and 200 μL of this (plasma-treated medium) was then used to replace the medium on the cells in the 96-well plate.

previously.\textsuperscript{11,12} The plasma unit consisted of three regions: the gas diffusion region, the main discharge region, and the radical transportation region. While Ar gas was flowing, plasma in the main discharge region was excited by applying 10 kV from a 60-Hz commercial power supply to two electrodes that were 20 mm apart. The flow rate of Ar gas was set at 2 standard liters/min (slm), and the distance between the plasma source and the samples was fixed at L = 13 mm.

B. Cell Culture

The U251SP human glioblastoma brain tumor cell line was used. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% FBS and penicillin (100 U/mL)-streptomycin (100 μg/mL). Primary normal human brain astrocytes (ACBRI-371) were purchased from the Applied Cell Biology Research Institute (ACBRI, Kirkland, WA, USA). The cells were grown in CSC complete recombinant medium supplemented with Culture Boost (Cell Systems Corporation, Kriklan, WA, USA). Attachment factor was used to treat the flask before cells were seeded (Cell Systems). All cells were maintained in a humidified incubator with 5% CO\textsubscript{2} at 37°C.
C. Cell Proliferation Assay (MTS Assay)

Cells were seeded in 200 μL medium in a 96-well plate. On the following day, 3 mL fresh medium in a 6-well plate was treated with plasma (L = 13 mm, 2.0 slm), and 200 μL of this (PAM) was used to replace the medium on the cells in the 96-well plate. On the following day, cell viability was assayed using the Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Absorbance was then measured at 490 nm with a Microplate Reader (POWERSCAN HT; DS Pharma Biomedical, WA, USA). The absorbance values were averaged over three independent experiments, and error bars show the mean ± SEM.

D. Cell Size Analysis

Cells were plated at a density of 1 × 10^5 cells in 3 mL medium in a 6-well plate. On the following day, PAM was produced as described above, and this (PAM) was used to replace the medium on the cells in the 6-well plate. On the following day, cell size was measured with a Scepter counter (Millipore, Temecula, CA, USA) according to the manufacturer’s instructions.

E. Apoptosis

Cells were seeded in 200 μL medium in an 8-well culture slide. On the following day, PAM was produced as described above, and 200 μL of this (PAM) was used to replace the medium on the cells in the 8-well culture slide. Two hours after treatment with PAM, the Cell Event Caspase 3/7 detection reagent (5 μM, Invitrogen, Carlsbad, CA) were added and continuously incubated for 2 h at 37°C. Cells were observed using a Keyence microscope.

F. Western Blot Analysis

Cells were plated in 3 mL medium on a 6-well plate. On the following day, PAM was produced as described above, and this PAM was used to replace the medium on the cells in the 6-well plate. Four hours after treatment with PAM, cells were lysed with RIPA Lysis Buffer (Millipore) containing protease inhibitors for 10 min on ice. Protein concentrations were determined using the Bradford Reagents (Sigma). Total proteins (20 μg) were loaded onto 4–12% Nupage Bis-Tris gels (Life Technologies, Grand Island, NY, USA) and transferred electrophoretically to polyvinylidene fluoride membranes using iBlot (Life Technologies). Membranes were blocked with Block Ace (DS Pharma Biomedical, Tokyo, Japan) for 1 h at room temperature and then incubated with anti-total AKT (1:1000, Cell Signaling Technology, Danvers, MA, USA) and anti-phosphorylated AKT (Ser 473) (1:1000, Cell Signaling Technology) and β-actin (1:10000, Abcam, Tokyo, Japan) antibodies and subsequently washed with T-PBS. Then membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Blotted proteins
were prepared using enhanced chemiluminescence reagent (GE Healthcare Bio-Science KK, Tokyo, Japan) and visualized with ImageQuant LAS 4000 (GE Healthcare Bio-Science KK).

III. RESULTS

A. PAM Killed Glioblastoma Brain Tumor Cells

In this study, we used PAM rather than directly treating cells with plasma because of two technical advantages. First, we were able to simplify the experimental designs. When we treat cancer cells in medium, the cells are directly affected by the plasma and indirectly by PAM. By creating PAM in the absence of cells and then adding it to cells, we eliminated any possible direct effects of plasma. Plasma generally produces ions, electrons, free radicals, and light such as ultra violet and vacuum ultra violet. Using PAM, we eliminated any possible direct effects of light on the cells. Second, rather than treating each sample one by one, we were able to simultaneously treat multiple samples, which reduces experimental errors. PAM was prepared using our NEAPP, as previously described in detail [8, 9]. Additional information is provided in the materials and methods.

A diagram depicting the experimental setup is shown in Figure 1. The flow rate of Ar gas was set at 2.0 standard liter/min (slm), and the distance between the plasma source and the samples was fixed at L = 13 mm. In all experiments, 3 mL fresh medium in a 6-well plate was treated with plasma for 1, 3, or 5 min, and PAM was then used to replace the medium on cells in either a 96-well plate or a 6-well plate.

To investigate the anti-tumor effects of PAM on U251SP glioblastoma cells, 1000, 5000, or 10000 cells were plated in a 96-well plate, and 200 µL PAM was used to replace the medium. The number of living cells was counted following a cell proliferation (MTS) assay. When treated with medium exposed to plasma (i.e., PAM) for 1 min, 1000 U251SP glioblastoma cells were effectively killed by PAM, whereas control (untreated and Ar gas only) cells were not damaged. However, when 5000 or 10000 cells were treated with PAM, no cell death was seen (Figure 2). These results suggest that PAM induced cell death not because the plasma destroyed essential materials for cell survival such as nutrients in the medium, but because plasma produced some anti-tumor materials in the medium. When the medium was treated with plasma for 3 or 5 min, even 10,000 cells were effectively killed by PAM (Figure 2). These results suggest that the anti-tumor effects on glioblastoma cells depend on plasma duration. We also measured the duration of the effectiveness of PAM (Figure 3). PAM was stored for 1, 8, or 18 h before using it to replace the medium on the cells. PAM stored for 1 or 8 h after plasma treatment showed anti-tumor effects on U251SP glioblastoma cells. However, PAM stored for 18 h after plasma treatment did not, indicating that PAM lost the anti-tumor effects by 18 h after plasma treatment. These results also suggest that the anti-tumor effects in medium were not due to the plasma destroying cell survival factor(s), but rather were due to production of cell death factor(s). Thus, the duration of the effectiveness of PAM was between 8 and 18 h. The components in the medium that have such a long
B. PAM Induced Apoptosis on Glioblastoma Brain Tumor Cells

One reason that plasma is recognized as a promising tool for cancer therapy is because plasma induces apoptosis in several types of cancer cells. Induction of apoptotic cell death is beneficial compared to induction of necrosis, another type of cell death, because necrosis often causes inflammation. To investigate whether PAM induces apoptosis in glioblastoma cells, we observed the morphology and activation of Caspase3/7 of glioblastoma cells after PAM treatment (Figure 4). Four hours after PAM treatment, most cells showed changes in cell shape, and activation of Caspase-3 and -7 were detected. Cells were small and round, and cell nuclei were aggregated. These morphological changes and activation of Caspase-3 and -7 are typical of apoptosis. We also measured
cell sizes using a counter based on the Coulter principle (Figure S1). PAM-treated cells indeed became smaller than control (untreated and Ar gas only) cells, suggesting the possibility of apoptosis.

C. PAM Selectively Killed Glioblastoma Brain Tumor Cells

Selectivity is one of the most important aspects in cancer therapy. We previously showed that our NEAPP selectively kills ovarian cancer cells but not normal fibroblast cells. In this study, we chose astrocytes as normal cells to compare them to glioblastoma brain tumor cells. We compared proliferation of primary normal human ACBRI-371 astrocytes to U251SP glioblastoma cells using the MTS assay. When medium was treated with plasma for 3 min, 10,000 ACBRI-371 cells were unaffected by PAM, whereas the same number of U251SP cells were killed by PAM (Figure 5, p = 0.000766), showing that our PAM selectively killed glioblastoma brain tumor cells but not normal astrocytes.

D. PAM Downregulated the Expression of AKT Kinase in Glioblastoma Brain Tumor Cells

Intracellular molecular mechanisms from plasma to cell death (apoptosis) are a fascinating frontier in plasma medicine. Currently, some researchers recognize that
apoptosis is a key event in plasma-induced cancer cell death. However, the signal transduction pathways responsible for cancer cell death remain unclear. One of the most important signal transduction pathways for cancer cell survival is the PI3K/PTEN-AKT pathway. This pathway is constitutively activated because of the absence of PTEN function in most human glioblastoma brain tumor cells, including U251SP cells. We reasoned that glioblastoma cells treated with PAM may show downregulation of PI3K/PTEN-AKT pathway activity, leading to cell death. To address this hypothesis, we performed western blot analysis to detect the expression and activity of AKT kinase, a marker molecule of cell survival signals. PAM-treated cells showed downregulation of both total AKT expression and phosphorylated AKT (at Ser 473) compared to the expression of phosphorylated (at Ser 473) and total AKT in control (untreated and Ar gas only) cells (Figure 6). AKT activation is partially responsible for inhibiting apoptosis, suggesting that apoptosis was induced in PAM-treated cells.
through downregulation of the activity of the PI3K/PTEN-AKT signal transduction pathway.

IV. DISCUSSION

Although it is not clear what factor(s) in the plasma-activated solution induces anti-tumor effects, plasma-activated solutions including plasma-activated water and plasma-activated medium are valuable because of anticipated medical applications in cancer therapy for surgically intractable disease such as peritoneal metastasis and meningeal dissemination. Our results suggest that the duration of the effectiveness of PAM is between 8 and 18 h. In terms of medical applications, this lifetime may be acceptable. Based on two observations, we conclude that our NEAPP produced some factor(s) in the medium that produced anti-tumor effects on glioblastoma cells and that our NEAPP did not destroy essential components in the medium. First, when medium was treated with plasma for 1 min, 5000, and 10,000 U251SP cells were not killed by PAM. Second, when PAM was incubated for 18 h, the anti-tumor effects of PAM were lost, but the growth effects of the medium were retained. One possibility

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**FIGURE S1.** PAM induced apoptosis. U251SP glioblastoma cells were plated at a density of $1 \times 10^5$ cells in 3 mL medium in a 6-well plate. On the following day, 3 mL fresh medium in a 6-well plate was treated with plasma, Ar gas, or was untreated for 5 min (L = 13 mm, 2.0 slm), and this (plasma-treated medium) was used to replace the medium on the cells in the 6-well plate. On the following day, cell size was measured.
is that \( \text{H}_2\text{O}_2 \) produced by plasma in the medium may be responsible for the anti-tumor effects,\(^\text{15}\) but an \( \text{H}_2\text{O}_2 \)-independent mechanism may also exist.\(^\text{10}\) The factor(s) that induce the anti-tumor effects and that have a lifetime of 8 to 18 h remain to be elucidated in future studies.

**FIGURE 5:** PAM effectively killed glioblastoma cells, but not normal astrocytes. U251SP glioblastoma cells and ACBRI-371 astrocytes were plated at a density of \( 1 \times 10^4 \) cells in 200 \( \mu \text{L} \) medium on a 96-well plate. On the following day, 3 mL fresh medium in a 6-well plate was treated with plasma, Ar gas, or was not treated for 3 min (\( L = 13 \text{ mm}, 2.0 \text{ slm} \)), and 200 \( \mu \text{L} \) of this (PAM) was used to replace the medium on the cells in the 96-well plate. On the following day, cell viability was assayed using the MTS assay. **p < 0.001.

**FIGURE 6:** PAM downregulated total AKT kinase expression and phosphorylated AKT in glioblastoma cells. Western blotting of total AKT and phosphorylated AKT (at Ser473) was performed on U251SP cells. Cells were treated with PAM or control (Ar gas and untreated) medium. \( \beta \)-actin was used as a loading control.
Researchers in the field of plasma medicine appreciate that plasma induces apoptosis in cancer cells because apoptosis does not lead to inflammation. However, the molecular mechanisms occurring in cells from plasma to apoptosis remain to be discovered. In this study, we showed that glioblastoma brain tumor cells also underwent apoptosis following PAM treatment. In addition, we showed that a key molecule in the survival signal transduction pathway (the PI3K/PTEN-AKT pathway), AKT kinase, was downregulated by PAM treatment. Most cancer cells including U251SP glioblastoma tumor brain cells, which have loss-of-function mutations in PTEN, express high AKT kinase activity, leading to aberrant, uncontrolled cell growth. Our finding that PAM downregulated the expression of total AKT kinase and phosphorylated AKT kinase suggests potential possibilities for therapy of glioblastoma brain tumors and other cancers that show overactivation of the PI3K/PTEN-AKT pathway.

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