Cell survival and proliferation signaling pathways are downregulated by plasma-activated medium in glioblastoma brain tumor cells

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ABSTRACT: We previously reported that plasma-activated medium (PAM) selectively kills glioblastoma brain tumor cells by downregulating the signaling molecule, the serine-threonine kinase AKT. AKT kinase plays a key role in survival and proliferation by acting as a hub molecule in the signaling network to inhibit apoptosis. The pathways that contain AKT and that are affected by PAM are unclear. In this study of glioblastoma brain tumor cells, phosphorylation of AKT at both Ser473 and Thr308 was downregulated by PAM, suggesting that upstream signaling by the mammalian target of rapamycin complex 2 (mTORC2) and phosphatidylinositol-3 kinase (PI3K)/3-phosphoinositide-dependent protein kinase-1 (PDK1) were affected by PAM. Furthermore, the extracellular regulated kinase (ERK) signaling pathway, which is parallel to the AKT signaling pathway, was downregulated by PAM, and the mTORC1 signaling pathway, which is a major downstream signaling pathway of AKT and ERK, was also downregulated by PAM. In addition, CD44, a cell membrane-bound receptor that promotes both the AKT pathway and the ERK pathway, was downregulated by PAM. Taken together, these results suggest that PAM completely downregulates the survival and proliferation signaling network in glioblastoma brain tumor cells.

I. INTRODUCTION

Recently, applications of non-thermal plasma (NTP) in the medical field, also called plasma medicine, have emerged as a distinct field because many important effects such as wound healing, blood coagulation, and promotion of regeneration have been reported to be mediated by NTP.1–5 NTPs can also be beneficial in cancer therapy, and we...
and other researchers have developed our own NTP applications in cancer therapy. Researchers in the field of plasma medicine have realized that plasma-activated solutions may have anti-tumor effects on cancer cells. These findings have broadened the potential use of plasma in medicine, but the mechanisms that lead to apoptosis in cancer cells by plasma remain to be elucidated. Plasma induces the production of intracellular reactive oxygen species and activates DNA damage signaling, including p53 expression. Other important mechanisms that lead to apoptosis are survival and proliferation signaling pathways that inhibit apoptosis in cancer cells. We previously reported that glioblastoma multiforme (GBM) brain tumor cells are selectively killed by plasma-activated medium (PAM) via induction of apoptosis by downregulation of the key signaling molecule of survival and proliferation, AKT kinase. These results suggest that PAM may be used in powerful chemotherapies. However, the details of the effects of PAM on survival and proliferation signaling pathways in cancer and normal cells must be investigated prior to application.

The AKT, extracellular regulated kinase (ERK), and mammalian target of rapamycin (mTOR) signaling pathways have been shown over the past decades to play key roles in the transmission of survival and proliferation signals from membrane-bound receptors. Elevated levels of activated components of these pathways are often associated with poor prognosis in cancer patients. ERK is a mitogen-activated protein kinase (MAPK) that is a member of the widely conserved family of serine/threonine protein kinases, which are involved in many cellular programs such as cell proliferation and survival. Upon stimulation, a sequential three-part protein kinase cascade is initiated, consisting of a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase. The serine/threonine kinase AKT plays an essential role in various cellular processes, including cell growth and proliferation, metabolism, and cell survival. The importance of the AKT pathway is highlighted by the mutation of various components of the pathway such as the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and phosphoinositide-3 kinase (PI3K) in human cancers. Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that is conserved across eukaryotic species and is a critical effector in cell signaling pathways that are commonly dysregulated in human cancers. mTOR regulates cell growth by controlling mRNA translation, ribosome biogenesis, autophagy, and metabolism. mTOR interacts with several proteins to form two distinct complexes called mTOR complex1 (mTORC1) and 2 (mTORC2). mTORC1 is composed of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (MLST8), and the recently identified partners PRAS40 and DEPTOR. mTORC2 is composed of mTOR, rapamycin-insensitive companion of mTOR (RICTOR), MLST8, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1). mTORC1 integrates the upstream signaling pathways of ERK and AKT. mTORC2 phosphorylates AKT at Ser473 and is critical to the AKT signaling pathway.

Malignant gliomas are the most common primary brain tumor in adults. The prognosis for patients with these tumors remains poor despite advances in diagnosis.
and standard therapies such as surgery, radiation therapy, and chemotherapy. Activation of AKT, ERK, and mTOR plays an important role in glioma formation and progression. Thus, AKT, ERK, and mTOR are attractive targets for cancer therapy as a means of reducing the apoptotic threshold and preferentially killing cancer cells. Thus, the finding that PAM downregulates AKT in GBM sheds new light on cancer therapy for GBM.

In this study, we investigated the signaling pathways that were responsible for the lack of survival and proliferation of GBM cells in the presence of PAM. We found that expression of both AKT and ERK was downregulated by PAM. In addition, the mTORC1 signaling that integrates the AKT and ERK signaling pathways was also downregulated by PAM. Expression of the membrane-bound receptor CD44, which regulates both AKT and ERK signaling pathways, was also downregulated by PAM. Taken together, these results show that components of the major signaling pathways involved in proliferation and survival were downregulated by PAM in GBM cells.

II. MATERIALS AND METHODS

A. Experimental setup for producing PAM

A diagram depicting the experimental setup is shown in Fig. 1. Approximately $3 \times 10^5$ cells were plated in 3 mL medium on a 6-well plate. On the following day, NTP with ultra-high electron density (approximately $2 \times 10^{16}$ cm$^{-3}$) was produced with an estimated atomic oxygen radical density of approximately $4 \times 10^{15}$ cm$^{-3}$, as described previously. 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 L min$^{-1}$ (slm), and the distance between the plasma source and the samples was fixed at $L = 7$ mm. Four milliliters of medium in a 60-mm dish was treated with the NTP, 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, Billerica, MA, USA) containing protease inhibitors for 10 min on ice for western blot analysis.

B. Cell culture

The U251SP human glioblastoma brain tumor cell line was used. WI-38 cells, which are normal fibroblasts, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS and penicillin (100 U mL$^{-1}$)-streptomycin (100 μg mL$^{-1}$; P/S). All cells were maintained in a humidified incubator with 5% CO$_2$ 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, Kirkland, WA, USA). The absorbance values were averaged over three independent experiments, and data are expressed as means ± SEM.

D. Western blot analysis

Protein concentrations of the cell lysates were determined using Bradford Reagents (Sigma). Total proteins (20 μg) were loaded onto 4~12% Nupage Bis-Tris gels (Life Technologies, Carlsbad, CA, USA) and transferred electrophoretically to polyvinylidene fluoride membranes. Membranes were blocked with Block Ace (DS Pharma Biomedical) for 1 hour at room temperature and then incubated with anti-total AKT, anti-phosphorylated AKT (Ser473), anti-phosphorylated AKT (Thr308), anti-total ERK1/2, anti-phosphorylated ERK1/2 (Thr202/Tyr204), anti-total mTOR, anti-phosphorylated mTOR, anti-total p70S6K, and anti-phosphorylated p70S6K antibodies. Signals were detected using an enhanced chemiluminescence detection system (Millipore, Billerica, MA, USA) and visualized on a Gel Doc apparatus (Bio-Rad, Hercules, CA, USA).
mTOR (Ser2448), anti-total S6K, anti-phosphorylated S6K (Thr389), anti-total EGFR, anti-CD44 (1:1000, Cell Signaling Technology, Danvers, MA, USA), and β-actin (1:10,000, Abcam, Tokyo, Japan) antibodies and subsequently washed with T-PBS. Then, membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Blotted proteins were detected using enhanced chemiluminescence reagent (GE Healthcare Bio-Science KK, Tokyo, Japan) and visualized with ImageQuant LAS 4000 (GE Healthcare Bio-Science KK).

E. Immunostaining

Cells were seeded in 200 μL medium in an 8-well culture slide. On the following day, 3 mL fresh medium (DMEM with 10% FBS, 1% P/S) in a 6-well plate was treated with plasma for 3 min (L = 13 mm, 2.0 slm), and 200 μL of this PAM was used to replace the medium on the cells in the 8-well culture slide. Four hours after treatment with PAM, cells were fixed with 4% paraformaldehyde for 20 min at room temperature and blocked with 10% normal goat serum and 0.1% Triton X-100 in PBS for 1 hour. Cells were then probed with anti-CD44 antibody (1:100, Cell Signaling Technology) and incubated for 24 hours at 4°C. Cells were incubated with an Alexa488-conjugated secondary antibody for 1 hour, incubated with rhodamine phalloidin for 20 min at room temperature, and embedded in DAPI-containing mounting medium. Fluorescence was detected using a Keyence microscope.

III. RESULTS

A. PAM selectively killed U251SP GBM cells compared to WI-38 normal fibroblast cells.

We previously showed that PAM selectively kills U251SP GBM cells compared to normal astrocyte cells. The safety of PAM will be important when treating humans; therefore, we also investigated the selectivity of killing by PAM of U251SP GBM cells compared to WI-38 normal fibroblast cells using a survival assay (Fig. 2). When medium was treated with NTP for 3 min, PAM effectively killed GBM cells but did not kill WI-38 normal fibroblast cells.

B. PAM downregulated phosphorylation of AKT at both Ser473 and Thr308 in GBM cells

We previously reported that PAM selectively kills GBM cells by downregulating the survival and proliferation signaling molecule, AKT kinase. To determine which pathways upstream of AKT are affected by PAM, we examined the expression levels of phosphorylated AKT at Ser473 and Thr308. AKT is phosphorylated at Ser473 by mTORC2 and at Thr308 by 3-phosphoinositide-dependent protein kinase-1 (PDK1), which is downstream of PI3K (Fig. 3A). Activation of PI3K results in phosphatidylinositol-
sitol (3,4,5)-trisphosphate (PIP3) synthesis generated by phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP2). PTEN antagonizes PI3K by converting PIP3 back to PIP2 by dephosphorylation of PIP3. PDK1 is activated by the presence of PIP3. Phosphorylation of AKT at both Ser473 and Thr308 are critical for promoting survival/proliferation. PAM downregulated phosphorylation of AKT at both Ser473 and Thr308, suggesting that both mTORC2 signaling and PI3K/AKT signaling were downregulated by PAM (Fig. 3B). Expression levels of AKT were also investigated in WI-38 normal fibroblast cells (Fig. 3B). As we expected, PAM did not downregulate the expression levels of AKT in WI-38 cells. Phosphorylation of AKT at Ser473 was not detected, and very low phosphorylation of AKT at Thr308 was detected.

C. PAM downregulated the ERK signaling pathway, which is parallel to the AKT signaling pathway, in GBM cells

Two major signaling pathways exist that connect membrane-bound receptors to proliferation and survival (Fig. 4A). One is the PI3K/PDK1/AKT pathway and the other is the Raf/MEK/ERK pathway. ERK is a downstream component of an evolutionarily conserved signaling module that is activated by the Raf serine/threonine kinases. Raf activates MAPK/ERK kinase (MEK)1/2 dual-specificity protein kinases, which then activate ERK1/2.

To investigate whether the ERK signaling pathway is affected by PAM, we examined the expression levels of phosphorylated ERK. ERK is phosphorylated at Thr202 and Tyr204 by MEK. PAM downregulated the expression of phosphorylated ERK1/2,
whereas expression levels of total ERK1/2 were not affected, suggesting that PAM downregulated the Raf/MEK/ERK signaling pathway that is responsible for proliferation and survival (Fig. 4B). As we expected, little phosphorylated ERK1/2 was detected in WI-38 fibroblast cells (Fig. 4B).

D. PAM downregulated the mTOR signaling pathway that integrates the AKT and ERK signaling pathways in GBM cells

mTORC1 integrates the AKT and ERK signaling pathways to promote proliferation and survival signals (Fig. 5A). Both AKT and ERK signaling pathways were downregulated by PAM, suggesting that mTOR signaling is also downregulated by PAM. To test this hypothesis, we investigated the expression levels of mTORC1 signaling molecules (Fig. 5B). Phosphorylation of mTOR at Ser2448 was downregulated by PAM, whereas total expression levels of mTOR were not affected by PAM. In addition, phosphorylation of the downstream signaling molecule of mTORC1, p70S6 kinase (S6K), at Thr389 was downregulated by PAM, whereas total expression levels of S6K were not affected by PAM. These results suggest that the mTORC1 signaling events that are downstream of AKT and ERK signaling were consistently downregulated by PAM. PAM did not affect mTORC1 signaling in WI-38 fibroblast cells (Fig. 5B).
We examined whether the autophagy signaling pathway is activated by PAM. When the autophagy signaling pathway is activated, the expression levels of Beclin-1 and Atg5 are upregulated. PAM did not upregulate the expression levels of Beclin-1 or Atg5 (Fig. 6), suggesting that PAM did not induce autophagy.

E. PAM downregulated the membrane-bound receptor, CD44, which is upstream of the AKT and ERK signaling pathways in GBM cells

Various membrane-bound receptors transmit survival and proliferation signals to the AKT and ERK signaling pathways. CD44 is a major cell-surface hyaluronan receptor and is generally upregulated in human GBM. Lipid rafts on the cell-surface membrane bring the signaling molecules together to transmit CD44 signaling to both AKT and ERK (Fig. 7A). PAM downregulated the expression levels of CD44 (Fig. 7B).

To investigate whether membrane-bound CD44 was downregulated by PAM, we observed CD44 in single cells (Fig. 8). CD44 on the cell membrane was not detected in PAM-treated cells. Taken together, these results suggest that PAM downregulated expression of the membrane-bound receptor, CD44, which is upstream of the AKT and ERK signaling pathways.
IV. DISCUSSION

A. PAM downregulated major survival/proliferation signaling pathways in GBM cells

PAM downregulated expression of the main parallel survival/proliferation signaling pathways, the AKT and ERK signaling pathways. Consistent with these results, PAM downregulated the mTORC1 signaling pathway, which integrates the AKT and ERK signaling pathways. Chemical compounds that are anticipated to be useful in cancer therapy do not necessarily inhibit both AKT and ERK pathways. For example, resveratrol inhibits the AKT signaling pathway in GBM cells, but not the ERK signaling pathway. Moreover, PAM did not affect normal cells such as fibroblasts and astrocytes. These results suggest that PAM is a promising tool for cancer chemotherapy.

B. PAM downregulated expression of CD44, which is upstream of the AKT and ERK signaling pathways, in GBM cells

Clarifying the modes of action of cell death by PAM in terms of molecular mechanisms is important. In this study, we found that PAM downregulated expression of CD44,
which transmits survival and proliferation signals through the AKT and ERK pathways, in GBM cells. These results suggest that PAM attacks the cell membrane-bound receptor, CD44, on the GBM cell surface, which attenuates the survival and proliferation signaling pathways and leads to apoptosis. Other cell-membrane–bound receptors that are upstream of the AKT and ERK signaling pathways exist, such as receptor tyrosine kinases. Whether inhibition of those molecules is also part of the mechanism of cell death by PAM remains to be determined.

C. PAM induced rapid apoptosis without inducing autophagy

We previously reported that PAM induces apoptosis within 4 hours, because most cells were positive for cleaved Caspase-3/7 4 hours after adding PAM. In this study, we found that major survival/proliferation signaling pathways were downregulated 4 hours after PAM treatment, which is consistent with the fact that PAM induced apoptosis in GBM cells within 4 hours. However, PAM did not induce autophagy. Oxidative stress is responsible for plasma-induced apoptosis. H$_2$O$_2$ induces autophagy and apoptosis in GBM cells. The differences in responses to PAM and H$_2$O$_2$ suggest that cell death mediated by PAM cannot be explained only by the H$_2$O$_2$ produced by plasma treatment. One possible reason that PAM induced apoptosis but not autophagy is that PAM may rapidly and strongly switch on the program of apoptosis before it activates the program

![Western blotting for Beclin-1 and Atg5](Image)

**FIG. 6:** PAM did not affect autophagy signaling in GBM cells. Western blotting for Beclin-1 and Atg5 was performed with U251SP cells and WI-38 cells. Cells were treated with PAM or control (Ar gas and untreated) medium. β-actin was used as a loading control.
of autophagy. Taken together, these results suggest that the mechanisms by which PAM induces cell death on cancer cells include apoptosis but not autophagy.

V. CONCLUSIONS

PAM downregulated the AKT, ERK, and mTOR signaling pathways, which mediate survival and proliferation by inhibiting apoptosis, in GBM cells. PAM also downregulated expression of the membrane-bound receptor, CD44, which activates those pathways. We believe that these findings provide significant insight into the intracellular molecular mechanisms of cell death of cancer cells mediated by PAM.

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FIG. 8: PAM downregulated CD44 expression on the cell surface in GBM cells. U251SP GBM cells were observed with a microscope 4 hours after treatment with PAM, Ar gas treated medium, or untreated medium, which replaced the medium on the cells. Scale bar represents 50 μm.

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