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Plasma-activated medium induced apoptosis on glioblastoma brain tumor cells by inhibiting growth/survival signaling.

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Abstract: Glioblastoma brain tumor cells and normal cells were treated with plasma-activated medium (PAM). Cell proliferation assays showed that glioblastoma cells were selectively killed by PAM. PAM induced apoptosis on glioblastoma. There are many signaling pathways that lead to apoptosis. We found that PAM downregulated the PI3K/PTEN-AKT pathway that is responsible for cell survival and proliferation on glioblastoma.

Keywords: PAM, glioblastoma, apoptosis, AKT

1. Background

Recently, applications of non-equilibrium atmospheric pressure plasma (NEAPP) in the medical field, also called plasma medicine, have become an emerging field because many important effects such as wound healing, blood coagulation, and promotion of regeneration have been reported to be mediated by NEAPP [1-3]. Some pioneering work has shown that NEAPP exerts anti-tumor effects and induces apoptosis in various cancer cells. NEAPP with ultrahigh electron density (approximately 2×10^{16} cm^{-3}) has been developed [4], and was previously applied to inactivate Penicillium digitatum spores [5] and as therapy for ovarian cancers [6]. Researchers in the field of plasma medicine have begun to study the fact that plasma affects cancer cells not only directly but also indirectly by changing the biological environment around the cells [7-9]. In other words, 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 [10]. It is often difficult to delineate tumor boundaries of glioblastomas. The current standard of care for patients with glioblastoma includes surgical resection followed by adjuvant radiotherapy and/or temozolomide chemotherapy. However, treatment is complicated by the location and invasive nature of the tumors and patient prognosis is poor. Thus, innovative cancer therapeutic strategies are needed to selectively kill glioblastomas.

Our previous results have shown that plasma-activated medium (PAM) could be a powerful tool for cancer therapy of glioblastoma [9], however, determining the molecular mechanisms of the anti-tumor effects of plasma on glioblastomas requires investigation.

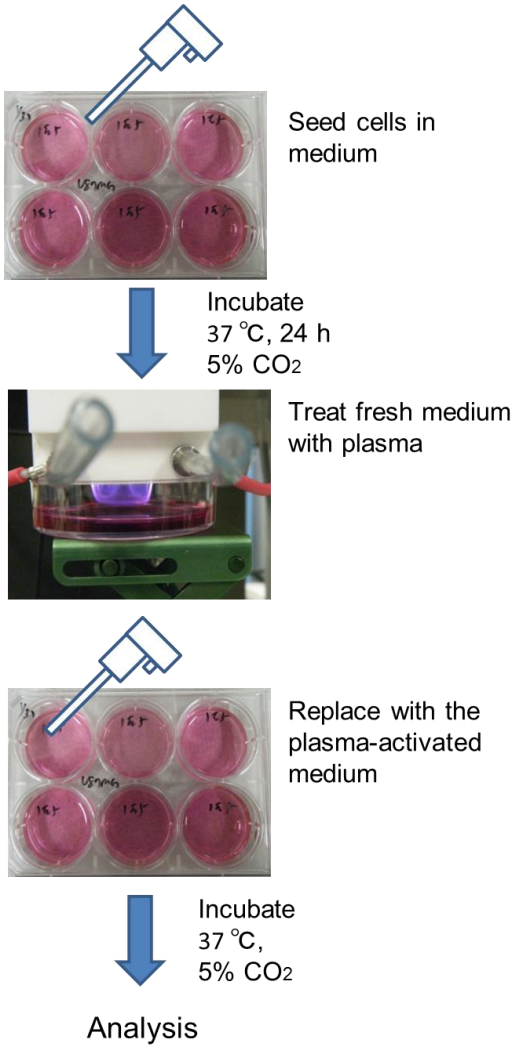

Fig. 1 The schematic of producing plasma-activated medium (PAM)

2. Results and Discussion

We used plasma-activated medium (PAM) rather than directly treating cells with plasma (Fig. 1). Gliomablastoma and fibroblast cells were plated at a density of 5000 cells in 200 μL of medium on a 96-well plate. On the following day, 3 mL of fresh medium (DMEM) in a 6-well plate was treated with plasma for 2 min (L = 13 mm, 2.0 slm), and 200 μL of them (plasma-treated medium) was replaced with the medium on the cells in the 96-well plate. On the following day, cell proliferation was evaluated by MTS assay. The cell proliferation assays showed that glioblastoma cells were effectively killed by the PAM, while fibroblast cells were not affected by the PAM (Fig. 2). These results suggest that the PAM selectively killed glioblastoma.

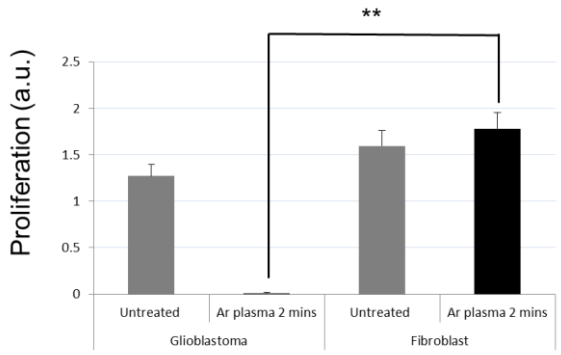

Fig. 2 Proliferation assay

In the previous study [9], PAM induced morphological changes consistent with apoptosis in glioblastoma cells and the cells decreased in size. In addition, an apoptotic molecular marker cleaved Capase-3/7 was detected in PAM treated cells. These results suggest that PAM induced apoptosis on glioblastoma.

There are many signaling pathways that lead to apoptosis through cleavage of Caspase-3/7. Our purposes are to figure out what signaling pathways are responsible for the apoptosis by PAM and understand the interactions between the PAM and intracellular components including membrane surface receptors, signaling molecules, genome, and so on.

One of the most important signal transduction pathways for glioblastoma cancer cell is the PI3K/AKT pathway (Fig. 3). Not only glioblastoma, but also many malignancies are known to be associated with abnormal activation of the PI3K-AKT pathway. The PI3K-AKT pathway is involved in regulating the signaling of multiple biological processes such as apoptosis, metabolism, cell proliferation, and cell growth [11]. Its abnormal activation, frequently observed in various types of cancer, leads to aberrant cell cycle progression, altered adhesion and motility, inhibition of apoptosis, and induction of angiogenesis. While mutations in AKT have not been observed in human gliblastoma, approximately 40% of glioblastoma tumors show mutation or loss of expression of the tumor suppressor gene PTEN, which functions as a major negative regulator of the PI3K/AKT signaling pathway.

Signaling through the PI3K/Akt pathway is initiated by stimuli from Growth factors that bind receptors in the cell membrane. These receptors include IGFR (Insulin-like Growth Factor Receptor), PDGFR (Platelet-Derived Growth Factor Receptor), EGFR (Epidermal Growth Factor Receptor), and so on.

Phosphatidylinositol (4,5)-bisphosphate (PIP2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3) are low-abundance membrane phospholipids that function in a number of crucial cellular processes, like membrane trafficking, plasma membrane-cytoskeleton linkages, second messenger signaling, cell adhesion and motility and regulation of proteins involved in phospholipid metabolism. These phosphoinositides direct two major independent signaling cascades. PIP3 is the effector of multiple downstream targets of the PI3K pathway. Activation of PI3K by growth factor stimulation of cells results in PIP3 synthesis generated by phosphorylation of PIP2.

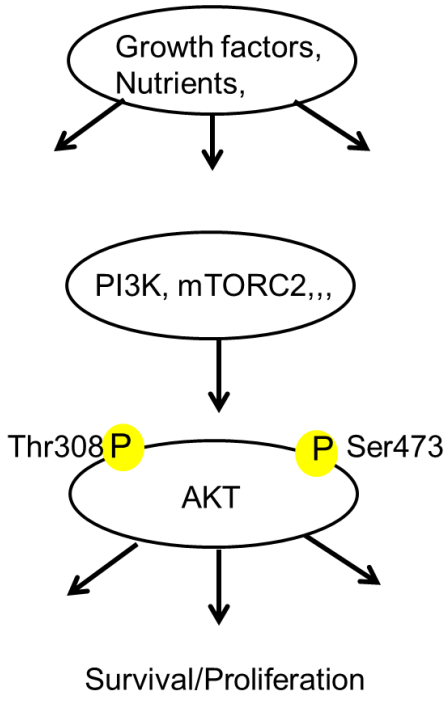

Fig. 3 The PI3K/AKT signaling pathway

AKT contains a pleckstrin homology (PH) domain which binds to PIP3 in the plasma membrane with high affinity. Once in correct position in the membrane, AKT can be phosphorylated by 3-phosphoinositide dependent protein kinase 1 (PDK1) at threonine 308 (Thr308) residue. Phosphorylation of serine 473 (Ser473) residue is a target of the mTOR complex 2 (mTORC2). Maximal

AKT activity is dependent on the phosphorylation status of both Thr308 and Ser473 residues.

To investigate whether the PI3K/AKT pathway is affected by PAM, the activity of AKT was measured by Western blotting analysis.

Glioblastoma and fibroblast cells were plated at a density of 3×10^5 cells in 6 mL of DMEM medium on a 6-well plate. On the next day, 4 mL of fresh medium (DMEM) in a 60 mm dish was treated with plasma for 5 min (L = 7 mm, 2.0 slm), and all of them (PAM) was replaced with the medium on the cells in the 6-well plate.

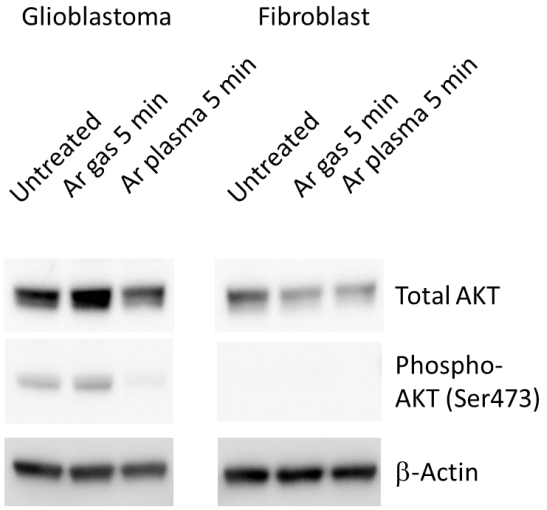

Fig. 4 PAM down-regulated the AKT signaling on glioblastoma.

Four hours after treatment with PAM, cells were lysed with RIPA Lysis Buffer containing protease inhibitors for 10 min on ice. Protein concentrations were determined using the Bradford Reagents. Total proteins (20 μg) were loaded onto 4~12% Nupage Bis-Tris gels and transferred electrophoretically to polyvinylidene fluoride membranes. Membranes were blocked with Block Ace for 1 h at room temperature and then incubated with anti-total AKT (1:1000) and anti-phosphorylated AKT (Ser 473) (1:1000) and β-actin (1:10000) antibodies and subsequently washed with T-PBS. Then, membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Blotted proteins were visualized using enhanced chemiluminescence reagent, and visualized with ImageQuant LAS 4000.

As shown in Fig. 4, 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 untreated cells. Phosphorylated AKT was not detected in fibroblast cells since the PI3K/AKT pathway was not over-activated in fibroblast cells (Fig. 4). The 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. Our finding that PAM downregulated the expression of total AKT kinase and phosphorylated AKT kinase suggests potential possibilities for therapy of glioma brain tumors and other cancers that show overactivation of the PI3K/PTEN-AKT pathway.

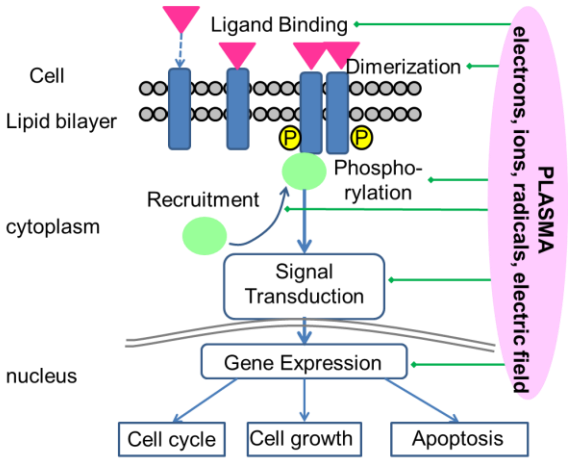

Fig. 5 Interactions between plasma and intracellular signaling networks

Our ultimate purpose is to figure out what interactions occur between plasma and cellular components such as signaling molecules (Fig. 5). Plasma interacts with gas phase and liquid phase to produce the secondary products that reach on and into cells. What products in gas and liquid phases critically affect cells are poorly understood. Those products might interact with cell surface receptor, lipid bilayer of the cell membrane, or directly any molecules in the cells. The fact that plasma induces apoptosis on cancer cells suggest that plasma somehow interacts with the signaling network on cells.

Previous studies have shown that the DNA damage and ROS production might be important to understand the interactions [8]. Our discovery that PAM downregulated the PI3K/AKT pathway on glioblastoma cells might provide new insights into the interactions between plasma and cells.

The modern pharmacology aims at designing the molecular target drugs for anti-cancer therapy so that it prevents the secondary effects based on the intracellular molecular mechanisms. This is why it is very important to understand what signaling pathways are affected by plasma and responsible for apoptosis of cancer cells.

Selective killing of cancer cells by PAM is another important aspect. There are some differences between cancer cells and normal cells in signaling networks. For example, the PI3K/AKT pathway is over-activated in cancer cells that harbor mutations in the pathway. PAM might attach the vulnerability in cancer cells based on the signaling

network. The molecular mechanisms of selective killing of cancer cells by plasma and/or PAM remain to be discovered.

4. Acknowledgements

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5. References

[1] Fridman G, Friedman G, Gutsol A, Shekhter AB, Vasilets VN, et al. (2008) Applied plasma medicine. Plasma Processes and Polymers 5: 503-533.

[2] Kong MG, Kroesen G, Morfill G, Nosenko T, Shimizu T, et al. (2009) Plasma medicine: an introductory review. New Journal of Physics 11.

[3] Morfill GE, Kong MG, Zimmermann JL (2009) Focus on Plasma Medicine. New Journal of Physics 11.

[4] Iwasaki M, Inui H, Matsudaira Y, Kano H, Yoshida N, et al. (2008) Nonequilibrium atmospheric pressure plasma with ultrahigh electron density and high performance for glass surface cleaning. Applied Physics Letters 92.

[5] Iseki S, Ohta T, Aomatsu A, Ito M, Kano H, et al. (2010) Rapid inactivation of Penicillium digitatum spores using high-density nonequilibrium atmospheric pressure plasma. Applied Physics Letters 96.

[6] Iseki S, Nakamura K, Hayashi M, Tanaka H, Kondo H, et al. (2012) Selective killing of ovarian cancer cells through induction of apoptosis by nonequilibrium atmospheric pressure plasma. Applied Physics Letters 100.

[7] Sato T, Yokoyama M, Johkura K (2011) A key inactivation factor of HeLa cell viability by a plasma flow. Journal of Physics D-Applied Physics 44.

[8] Kalghatgi S, Kelly CM, Cerchar E, Torabi B, Alekseev O, et al. (2011) Effects of Non-Thermal Plasma on Mammalian Cells. Plos One 6.

[9] Tanaka H, Mizuno M, Ishikawa K, Nakamura K, Kajiyama H, et al. (2011) Plasma-Activated Medium Selectively Kills Glioblastoma Brain Tumor Cells by Down-Regulating a Survival Signaling Molecule, AKT Kinase. Plasma Medicine 1: 265-277.

[10] Lee JJ, Kim BC, Park MJ, Lee YS, Kim YN, et al. (2011) PTEN status switches cell fate between premature senescence and apoptosis in glioma exposed to ionizing radiation. Cell Death and Differentiation 18: 666-677.

[11] (2010) On the Mechanism of Plasma Inducing Cell Apoptosis. Ieee Transactions on Plasma Science 38: 2451-2457.