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The strong anti-glioblastoma capacity of the plasma-stimulated lysine-rich medium

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Abstract

Plasma-stimulated medium (PSM) shows a remarkable anti-cancer capacity as strong as the direct cold atmospheric plasma (CAP) treatment of cancer cells. PSM is able to effectively resist the growth of several cancer cell lines. To date, the sole approach to strengthen the anti-cancer capacity of PSM is extending the plasma treatment time. In this study, we demonstrated that the anti-glioblastoma capacity of PSM could be significantly increased by adding 20 mM lysine in Dulbecco’s modified Eagle’s medium (DMEM). This study provides clear evidence that the anti-glioblastoma capacity of PSM could be noticeably enhanced by modifying the composition of medium without increasing the CAP treatment time.

Keywords: cold plasma, cancer treatment, media, lysine

(Some figures may appear in colour only in the online journal)

Introduction

The anti-cancer capacity of the cold atmospheric plasma (CAP) treatment has been extensively studied over the past decade [1–3]. The CAP treatment shows its strong selective killing effect on dozens of cancer cell lines [4–6]. Most of CAP treatment is performed by using plasma to directly treat cancer cells [7] or tumor tissues [8]. Recently, Tanaka et al [9] and Yan et al [10, 11] found that the Plasma-stimulated medium (PSM) made by the plasma treatment on culture medium was able to kill many cancer cell lines as effectively as the direct CAP treatment did. In addition, PSM also shows its anti-cancer capacity in vivo. Utsumi et al have injected PSM into the tumor in mice and found that the growth of tumor was significantly inhibited [12]. PSM can be stored for several days under an appropriate condition [10, 13], which makes it to be a convenient anti-cancer method at some special circumstances such as the moment when a CAP device is not available. PSM is a promising pharmaceutical anti-cancer approach.

So far, it was shown that the anti-cancer capacity of PSM could only be enhanced by extending the plasma treatment time [10, 13]. Nonetheless, the recent study revealed that the fetal bovine serum (FBS) [10] in whole medium and the pyruvate [13] in some specific DMEM would eliminate the anti-cancer performance of PSM. To date, these results are the only initial clues about regulating the anti-cancer capacity of PSM by changing the composition of medium. However, due to the growth factor independence feature of cancer [14], FBS is not necessary for the growth of cancer cells in culture. In addition, many commercialized DMEM actually does not contain pyruvate. Thus, in most cases, just using the FBS-free medium or the pyruvate-free DMEM will not optimize the anti-cancer capacity of PSM.
Glioblastoma multiforme (World Health Organization grade IV astrocytoma) is the most lethal brain tumor in adults [15]. Currently, a median survival of the glioblastoma multiforme patients who have been treated with standard surgical resection followed by radiation therapy and chemotherapy will be just about 1 year [16]. Recently, CAP displayed a strong anti-glioblastoma capacity on glioblastoma cells in vitro [9, 17, 18] and tumor tissues originated from the hypodermal planted glioblastoma cells in mice [17, 19]. As such CAP has promising application in the future glioblastoma treatment.

In this study, we first demonstrated that the anti-glioblastoma capacity of PSM could be significantly enhanced by adding particular amino acids in DMEM. Gold nanoparticles have been used to enhance the anti-cancer capacity of the direct CAP treatment on melanoma cells [20] and glioblastoma cells [21]. However, the cost of gold nanoparticles is much higher than amino acids. Thus, our study provided a low cost method to optimize the anti-cancer application of PSM.

Figure 1. A schematic illustration for the general research strategy.

Figure 2. Conventionally, to achieve a noticeable anti-cancer performance over the glioblastoma cells with a high confluence, an adequately long plasma treatment is necessary. The data processing is illustrated in Methods.

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Methods

CAP device

In this study, the CAP device was a cold plasma jet generator using helium as the carrying gas [22]. It has been used to investigate the anti-cancer effect of PSM [10] and the physiological response of cancer cells after the direct plasma treatment [22, 23]. The plasma was generated between a central anode and a ring grounded cathode and was ejected out a quartz tube to vertically treat the DMEM in a 12-well plate. The helium gas flowed at a rate of 4.71 min$^{-1}$. The input voltage of DC power was 11.5 V. The output voltage was 3.16 kV. The gap between the outlet of quartz tube and the bottom of 12-well plate was 3 cm.

Medium and cell cultures

Standard DMEM (11965-118) was purchased from Life Technologies. No protein components or FBS were added in this DMEM. Such DMEM contains L-glutamine. In addition, all DMEM was mixed with 1% (v/v) antibiotic (penicillin and streptomycin) solution (Life Technologies) before any experiments. Human glioblastoma (U87MG) was provided by Dr Murad’s Lab at the George Washington University. All cancer cell lines were seeded with a designed confluence and were cultured in 100 μl of cell culture medium in each well on 96-well plate for 6 h under the standard cell culture conditions (a humidified, 37 °C, 5% CO2 environment). The cell culture medium used in the cell seeding process and initial cell culture process was composed of DMEM supplemented with 10% (v/v) fetal bovine serum (ThermoFisher Scientific) and 1% (v/v) antibiotic (penicillin and streptomycin) solution (Life Technologies). In each experiment, 6 wells in a single column on 96-well plate would be seeded with cancer cells. All wells on the margins of 96-well plate have not been used in experiments. Before using PSM to affect the growth of U87MG cells, these media which have been used to culture U87MG cells for 6 h were removed.

General research strategy

As illustrated in figure 1, 1 ml of DMEM was first treated by CAP in one well on a 12-well plate. After the treatment, 100 μl of the plasma-stimulated DMEM was transferred to culture U87MG cells seeded in one well on a 96-well plate for 6 h. The sample number for each case was 6. Thus, only 600 μl in each 1 ml of plasma-stimulated DMEM were transferred to culture U87MG cells. After that, U87MG cells were cultured
in the incubators for 72 h under a standard condition (a 37 °C, 5% (v/v) CO₂ and humidified environment). Ultimately, cell viability was qualified using MTT assay (Sigma-Aldrich).

**Measuring cell viability**

According to the protocols provided by manufacturer, the MTT assay was performed by following steps. First, 0.7 mg ml⁻¹ MTT-DMEM solution was prepared by dissolving MTT powder in standard DMEM. Second, the DMEM which had been cultured the treated cells for 72 h were removed from wells on 96-well plate. Third, U87MG cells were then cultured in MTT-DMEM solution under a standard culture conditions for 3 h. In each well, the volume of MTT-DMEM solution was 100 μl. Fourth, these MTT-DMEM in each well was replaced by 100 μl of 0.4% (v/v) hydrochloric acid containing isopropanol solution (OmniSolv). Fifth, the 96-well plate filled with hydrochloric acid containing isopropanol solution was read by a H1 microplate reader (Hybrid Technology) at a 570 nm of absorbance. To facilitate the formation of violet solution, the 96-well plate was shaken for 1 min before the measurement.

**Making the plasma-stimulated amino acids-rich DMEM and affecting the growth of U87MG cells**

We prepared 20 amino acids-rich DMEM. Each of them was prepared by dissolving specific purchased amino acids powders (Sigma-Aldrich) in DMEM. The DMEM used in this study was made by mixing 1% (v/v) penicillin-streptomycin (Life Technologies) with standard DMEM (11965-118, Life Technologies). For each case, 1 ml of specific amino acids-rich DMEM in a well of 12-well plate was treated by CAP for 1 min. After that, these plasma-stimulated amino acids-rich DMEM were transferred to affect the growth of U87MG cells immediately. The initial cell confluence during the seeding was 6 × 10⁴ cells ml⁻¹. U87MG cells were cultured for 6 h before the treatment. U87MG cells were cultured for 3 d before the cell viability was measured by MTT assay. The sextuplicate experiments were independently repeated for three times.

**Measuring H₂O₂**

H₂O₂ has been widely acknowledged as the main reactive species to cause the death of CAP-treated cancer cells [13, 24–26].
Thus, we qualified the H$_2$O$_2$ concentration in PSM using Fluorimetric Hydrogen Peroxide Assay Kit (Sigma-Aldrich) according to the protocols provided by manufacturer. In short, 50 $\mu$l of PSM was added into one well on a black clear bottom 96-well plate (Corning). Then, 50 $\mu$l of red peroxidase substrate stock solution (Sigma-Aldrich), 200 $\mu$l of peroxidase stock solution (Sigma-Aldrich), and 4.75 ml of assay buffer (Sigma-Aldrich) were mixed to prepare H$_2$O$_2$ probe solution. 50 $\mu$l of H$_2$O$_2$ probe solution was added in black 96-well plate and was mixed with 50 $\mu$l of PSM. After 30 min of storage at the room temperature protecting from light, a H1 microplate reader (Hybrid Technology) was used to measure the fluorescence with an excitation wavelength at 540 nm and an emission wavelength at 590 nm. The final measured fluorescent strength of the experimental group were obtained by deducting the measured fluorescent strength of the control group from the measured fluorescent strength of the experimental group. The standard H$_2$O$_2$ solution (Sigma-Aldrich) was used to prepare the standard H$_2$O$_2$ concentration-fluorescence curve. Based on this standard concentration-fluorescence curve, we obtained the H$_2$O$_2$ concentration in PSM. The triplicate experiments were independently repeated for three times.

**Data processing and statistical processing**

The data processing has two steps. First, the original experimental data about the cell viability was processed to be the relative cell viability through the division between the measured cell viability of U87MG cells cultured in PSM such as plasma-stimulated amino acids-rich DMEM to the measured cell viability of U87MG cells cultured in the untreated medium such as amino acids-rich DMEM. The measured cell viability of each case was equal to the mean value of 6 samples. And, as shown in figures 1 and A1, experimental data were finally shown as the mean ± s.d. of three independently repeated experiments. Second, it is necessary to point out that, for the amino acids-rich DMEM, we performed the experiment in 0 mM, 5 mM, 10 mM, and 20 mM amino acids-rich DMEM simultaneously for each amino acid case. Due to the experimental error, the relative cell viability of 0 mM case varies with the amino acids used in experiment (figure A1). Thus, to facilitate the comparison between different amino acids cases, we added a second step for data processing to unify the relative cell viability at 0 mM case from different amino acids groups to be 1. Specifically, the mean relative cell viability data (0 mM, 5 mM, 10 mM, and 20 mM) for each amino acid was normalized to the mean relative cell viability data of corresponding 0 mM case. Thus, the effect of amino acids on the anti-glioblastoma capacity of PSM is presented as the normalized relative cell viability. For the H$_2$O$_2$ data, the final results are the mean ± s.d. of three independently repeated experiments.

**Results and discussion**

*Only long CAP treatment can kill glioblastoma (U87MG) cells with a high confluence*

As Adachi *et al* found, PSM induced a confluence-dependent death of lung carcinoma cells (A549) [13]. We observed the similar phenomenon on the glioblastoma cells (U87MG) cultured in PSM for 3 d. 100 $\mu$l of U87MG cells solution with different confluences over $2 \times 10^4$ cells ml$^{-1}$ to $1 \times 10^5$ cells ml$^{-1}$ were seeded in 96-well plate for 6 h under the standard culture conditions. Then, 100 $\mu$l of plasma-stimulated DMEM was transferred to culture the seeded U87MG cells in a 96-well plate for 72 h before the final test on the cell viability. As shown in figure 2, the anti-glioblastoma capacity of PSM is inversely proportional to the seeding confluence of U87MG cells. One minute of cold plasma treatment only noticeably resists the growth of U87MG cells with a confluence lower than $4 \times 10^4$ cells ml$^{-1}$. To achieve a significant anti-cancer effect on U87MG cells with a confluence higher than $4 \times 10^4$ cells ml$^{-1}$, extending the treatment time is the sole solution.
The confluence-dependent trend indicates that single cancer cell should have a physiological threshold to tolerate the attack of the plasma-originated reactive species. Thus, when the cell confluence is higher, the dose of reactive species exerting on each cell is less. When each cell only touches the reactive species less than that physiological threshold, the anti-glioblastoma capacity of plasma-stimulated DMEM will be negligible. Based on above discussion, there are two strategies to achieve a better anti-glioblastoma capacity. One is increasing the generation of the plasma-originated reactive species by using a stronger plasma source or exerting a longer plasma treatment. Another option is decreasing the tolerable physiological threshold of the cancer cells to the attack of the reactive species in PSM by affecting the normal cells’ function synergistically using other methods; such as the synergistic treatment of nanoparticles and CAP on cancer cells [20, 21].

The plasma-stimulated lysine-rich DMEM is capable of noticeably decreasing the viability of glioblastoma (U87MG) cells

To achieve a noticeably enhanced anti-glioblastoma capacity of PSM over U87MG cells without extending the treatment time, we investigated the response of U87MG cells to the plasma-stimulated amino acid-rich DMEM. As shown in figure 3, the anti-glioblastoma capacity of the plasma-stimulated amino acids-rich DMEM significantly varies with not only the amino acids but also the concentration of amino acids in DMEM. Generally, cysteine, proline, aspartic acid, serine, glutamic acid, threonine, leucine, and glycine weaken the anti-glioblastoma performance of plasma-stimulated DMEM. In contrast, phenylalanine, alanine, histidine, arginine, tyrosine, and lysine enhance the anti-glioblastoma effect of plasma-stimulated DMEM. Other amino acids, including glutamine, methionine, asparagine, valine, isoleucine, and tryptophan, do not significantly affect the anti-glioblastoma capacity of plasma-stimulated DMEM. When the concentration of amino acids in DMEM are 5 mM, 10 mM, and 20 mM, tyrosine, arginine, and lysine most enhances the anti-glioblastoma capacity of the plasma-stimulated DMEM, respectively. And, among all experimental conditions, 20 mM plasma-stimulated lysine-rich DMEM shows the strongest anti-glioblastoma capacity. In contrast to the experiment performed in the standard DMEM, the relative cell viability of U87MG cells decreases 35% in the 20 mM plasma-stimulated lysine-rich DMEM. As a contrast, the relative cell viability of U87MG cells with a confluence of 6 × 10⁴ cells ml⁻¹ will only decrease 25% by just increasing the CAP treatment time from 1 min to 2 min (figure 2).

As high-resolution mass spectrometry revealed that oxidation and nitration occur on most cold plasma-treated amino acids [27]. Among them, slight oxidation is the main change on lysine [27]. However, the reaction between lysine and cold plasma is much weaker than that between methionine/cysteine and cold plasma [27]. Whether the slight oxidation on lysine after the cold plasma treatment will be toxic to U87MG cells is still unknown. At the moment, the chemical essence of anti-cancer capacity of CAP treatment is still unclear. Nonetheless, H₂O₂ has been acknowledged to be the main anti-cancer reactive species generated during CAP treatment [13, 24–26, 28]. To this end, the H₂O₂ generation in the plasma-stimulated lysine-rich DMEM was measured and is shown in figure 4. Obviously, in contrast to the standard DMEM, 20 mM lysine-rich DMEM actually decreases the H₂O₂ generation by 18.5%. Thus, the enhanced anti-glioblastoma capacity of plasma-stimulated lysine-rich DMEM may be due to other mechanisms rather than the increased H₂O₂ generation during CAP treatment.

The existing synergetic strategies for the CAP treatment, such as using gold nanoparticles to treat cancer cells before [20] or during [21] the plasma treatment, is based on a prerequisite that the auxiliary methods are toxic to cancer cells. The tolerable physiological threshold of cancer cells to the attack of reactive species may be reduced after these synergetic treatments. As a result, the anti-cancer capacity of CAP treatment will be enhanced. Lysine-rich DMEM may follow similar principle to achieve the strong anti-glioblastoma effect over U87MG cells, because 20 mM lysine-rich DMEM is toxic to glioblastoma cells (figure 5). To date, only limited study about the biological effect of lysine on cancer cells were performed. Only the antineoplastic activity of poly-L-lysine on ascites tumors in mice and HeLa cells has been reported [29]. Arnold et al proposed that the polylysine bind to the cancer cells’ membrane and induced a rapid leakage of small molecules across the cell membrane followed by rapid loss of RNA, DNA, and protein synthesis [29]. Despite the deep understanding on the antineoplastic mechanism is far from clear, the change on the cell membrane permeability still indicates that the transmembrane diffusion of the reactive species generated by CAP treatment may be facilitated and finally enhance the anti-cancer capacity on U87MG cells. Nonetheless, the biological effect of lysine on cancer cells may be quite different from the biological effect of poly-lysine on the same cell lines. Thus, further study is necessary to understand the enhanced anti-glioblastoma in the plasma-stimulated lysine-rich DMEM.

Conclusions

The plasma-stimulated medium is a novel anti-cancer tool in plasma medicine. In this study, we first demonstrate that the anti-glioblastoma capacity of PSM can be significantly enhanced by adding 20 mM of lysine in DMEM during the CAP treatment even the confluence of glioblastoma cells is high. The lysine has not increased the H₂O₂ generation during CAP treatment. The toxicity of 20 mM of lysine-rich DMEM over glioblastoma cells may causes strong anti-glioblastoma capacity of the plasma-stimulated lysine-rich DMEM. This study demonstrates that the anti-cancer capacity of PSM can be optimized by modifying the components of medium.

Acknowledgment

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Figure A1. The original experimental data for figure 3. Results are presented as the mean ± s.d. of three independently repeated experiments performed in sextuplicate. The data processing is illustrated in Methods.
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