Plasma enhance drug sensitivity to bortezomib by inhibition of cyp1a1 in myeloma cells

Dehui Xu¹, Qingjie Cui², Yuying Xu¹, Zeyu Chen¹, Wenjie Xia¹, Yanjie Yang³, Dingxin Liu¹

¹State Key Laboratory of Electrical Insulation and Power Equipment, Centre for Plasma Biomedicine, ²The School of Life Science and Technology, ³Department of Cardiovascular Medicine, First Affiliated Hospital of the Medical School, Xi’an Jiaotong University, Xi’an 710049, China

Background: Drug resistance is one of the major problems encountered in clinical therapy of multiple myeloma treatment. Combination treatment with several drugs may increase the sensitivity and overcome drug resistance.

Methods: Here, we combined chemotherapy with a newly developed technology, cold atmospheric plasma, to enhance drug sensitivity.

Results: We found that plasma treatment had a synergistic anti-cancer effect with a first line drug (bortezomib). Based on our previous study, we further found that plasma treatment could inhibit Notch pathway and down-regulate cyp1a1 expression and enzyme activity, which contributing to the enhanced drug sensitivity to bortezomib after combination of bortezomib with gas plasma.

Conclusions: Our results showed a new strategy to overcome drug resistance by combination of traditional chemotherapy with cold atmospheric plasma.

Keywords: Cold atmospheric plasma; cyp1a1; drug resistance; multiple myeloma (MM); notch

Submitted Jun 18, 2019. Accepted for publication Oct 11, 2019.
doi: 10.21037/tcr.2019.10.43
View this article at: http://dx.doi.org/10.21037/tcr.2019.10.43
Gas plasma could enhance the sensitivity to bortezomib in myeloma cells, by inhibiting Notch signaling and cyp1a1, which could be applied as a new combination treatment for a better anti-cancer effect and a lower drug side effect.

**Methods**

**Atmospheric pressure plasma jet (APPJ) generation system.**

In this study, CAP was produced by a plasma jet device, the schematic diagram of the device structure was shown in Figure 1A. It was modified based on a needle. An inner diameter of 4 mm stainless steel tube was used as a high-voltage electrode and gas inlet. The ground electrode was a 10 mm long copper sheet, which wrapped around the quartz tube at a distance of 10 mm from the nozzle. The plasma generation system is consist of a gas flow controller, a high-voltage power supply, oscilloscope, and the above mentioned APPJ device. It was powered by a 10 kHz sinusoidal power supply at 8 kV peak-to-peak voltage and the He gas flow was maintained at 2 SLM. The plasma plume could directly contact with the cell medium surface.

**Optical emission spectroscopy**

Optical emission spectra (OES) was detected by an Andor SR-750i grating monochromator (grating grooving 1,200 lines mm⁻¹) within a wavelength range of 300–800 nm. The optical fiber was oriented up the APPJ radial direction at a distance of 1 and 2 cm from the APPJ nozzle.

**Cell culture and plasma treatment**

LP-1 MM cell line was used in this study and the details could be found in our previous work (16). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal calf serum, 100 U/mL penicillin, and 50 µL/mL streptomycin (Corning, Ithaca, NY, USA) in an incubator (Thermo Scientific, Waltham, MA, USA) containing a humidified atmosphere of 5% CO₂ at 37 °C. Cells were refreshed 24 h before performing experiments. For plasma treatment, 2×10⁵ cells were cultured in 24 well plates in 300 µL RPMI1640 complete medium and were treated with plasma jet 1.5 cm away from the bottom of the plates. After treatment, cells were continually cultured for further experiments. Ethics approval was not required because no clinical experiments were involved in our paper.

**Cell viability assay**

In this study, we used CellTiter-Glo Assay kit (Promega, Madison, WI, USA) to detect the cell viability, which based on the quantitative determination of ATP in living cells. 100 µL of cells and 100 µL of Cell-Titer-Glo reagent were mixed into the opaque-walled multi-well plate, then cells were incubated at room temperature for 10 min. The luminescence was determined using the microplate reader (Thermo Scientific, USA) with the protocol of “Luminometric” measurement.
Extracellular and intracellular ROS detection

The ROS levels were monitored using CMH2-DCFDA (Invitrogen, USA) following the manufacturer’s instruction. After plasma treatment for 24 h, 10 µM of ROS dye was incubated with cells for 30 min at 37 °C. Extracellular ROS was measured with a microplate reader (Thermo Scientific) with excitation/emission at 485/530 nm using the protocol for “Fluorometric” measurement. Then, the cells were washed three times with PBS and collected for detection of intracellular ROS fluorescence by a fluorescence microscope (Olympus) with blue light motivating.

Real-time PCR analysis

Total RNA was extracted from cells by RNA kit II (Omega Bio-Tec Inc., USA) following manufacturer’s instructions and quantified with Nano Drop spectrophotometry (BioTek, USA). We used 2 µg total RNA to synthesize first strand cDNA by RevertAid first strand cDNA synthesis kit (Thermo Scientific). Real-time PCR was performed on Bio-Rad CFX Connect™ Real-time System (Bio-Rad, USA) and amplified with an optimized cycling condition: 5 min at 95 °C, then 10 s at 95 °C and 30 s at 60 °C for 38 cycles. The total reaction system was 20 µL: 10 µL 2× QuantiFast SYBR Green PCR MasterMix (Qiagen, Germany), 1 µL of cDNA templates, 0.5 µM primer and 8 µL of DNAase-RNase Free water. The primers were provided by Shenggong Company (Shanghai, China) and the sequences were used in our previous studies (8,17).

Cyp1a1 enzyme activity assay

Twenty four hours after plasma treatment for different time, cells were harvested and washed with PBS for 3 times. Then the cyp1a1 enzyme activity was measured by P450-Glo™ cyp1a1 Assay (Promega, USA) according to the manufacturer’s instructions.

Statistical analysis

All values are presented as mean ± SD of three independent experiments. Differences between groups were evaluated using one-way ANOVA and student T test. P<0.05 was considered statistically significant.

Results and discussion

Generation of He plasma by plasma jet

In this study, we generated the plasma through a device modified by a needle. The profile of the plasma jet device is shown in Figure 1A. He plasma was powered by a stainless steel tube with a voltage of 10 kHz/8 kV, which was also used as He gas injection. He gas flow was regulated at a ratio of 2 SLM. Figure 1B shows the discharging image of the He plasma jet.

Detection of emission spectra

Since there are many particles with high energy states in the plasma, by detecting their characteristic lines in the emission spectroscopy, the distribution of various particles in the plasma could be understood. The optical fiber was placed 1 and 2 cm away from the plasma jet and the emission spectra was shown in Figure 2. Several characteristic lines of N, He and O were marked in the spectra, and the emission intensity is negatively correlated with the distance from the plasma jet.

Plasma enhance the sensitivity to bortezomib

Bortezomib is a first line drug for MM clinical treatment, we investigated whether gas plasma has a synergistic effect with bortezomib in MM cells. The cell cytotoxicity of different concentration of bortezomib was tested by cell viability assay 24 and 48 h after co-incubation (Figure 3A). Meanwhile, the reduction of cell viability by plasma treatment for different durations was investigated 24 and 48 h after treatment (Figure 3B). We chose 1 and 3 nM concentration of bortezomib and 30 s of plasma treatment for synergistic effects analysis. After 24 h co-treated with bortezomib and plasma, we found that 30 s of plasma treatment could both significantly enhance the sensitivity to 1 and 3 nM of bortezomib treatment (Figure 3C).

Plasma increased ROS levels

Biological effects induced by plasma treatment were mostly related to ROS generation. We detected the extracellular and intracellular ROS accumulation after plasma treatment by micro-plate reader and fluorescence
It showed that the extracellular ROS level was increased by plasma treatment in a time dependent manner, while the ROS scavenger (NAC) could prevent the ROS accumulation by plasma treatment (Figure 4A). The intracellular ROS was also increased after plasma treatment as the fluorescent intensity was higher than the control and NAC could reverse it (Figure 4B).

**Inhibition of Notch and cyp1a1 by plasma treatment**

In our previous study, we have demonstrated that cyp1a1, a Cytochrome P450 enzyme for drug metabolism, is involved in bortezomib resistance in myeloma cells (8). Furthermore, Notch pathway is a critical signaling for regulating cyp1a1 activity. Therefore, we investigated whether plasma treatment had effects on these signaling. By real-time PCR we found that plasma treatment could significantly decrease the expression of Notch downstream target genes such as Hes1, Hes5, Hey1, Hey2 and HeyL (Figure 5A). Meanwhile, plasma treatment could down-regulate cyp1a1 expression in a time depend manner (Figure 5B). Besides, the enzyme activity assay showed that cyp1a1 activity was
Drug resistance is one of the major problems encountered in clinical therapy of multiple myeloma treatment. Bortezomib is a first line drug used in the standard treatment and has improved clinical outcome. However, some patients do not respond to bortezomib or they eventually relapse after response (18,19). Besides, the 5 years overall survival is still not satisfied and the combination of several drugs to overcome the multiple drug resistance is applied. In this study, we first tried to couple two different kinds of treatment: the chemotherapy and the plasma treatment, to enhance the anti-tumor effects. Gas plasma is a newly developed technology that has been widely applied in biological and medical applications. We found that gas plasma treatment and bortezomib had a synergistic effect on the induction of myeloma cell death, which may lower the drug concentration to avoid the drug side effects in the future. In previous study we found that suppressed by gas plasma treatment (Figure 5C).

Conclusions

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cyp1a1 up-regulated by Notch activation could induce drug resistance to bortezomib in myeloma cells (8), so we detected the Notch signaling and cyp1a1 expression after gas plasma treatment. Interesting, gas plasma treatment did suppress the Notch pathway and down-regulate the cyp1a1 expression and enzyme activity. But how the plasma interact with the Notch pathway remains unknown and it is an interesting work to be further investigated. As we know, plasma treatment induces various biological effects mostly through the generation of many ROS (11). It has been reported that Notch signaling could modulate ROS accumulation especially for H_{2}O_{2} (20,21). To the contrary, ROS could also regulate Notch pathway and affected cell metabolism and cell death (22). In our results, ROS produced by gas plasma discharging could suppress the Notch downstream target genes. Kim et al. reported that ROS could down-regulate Notch signaling and induce cell death in breast cancer cells (23). Cao et al. demonstrated that inhibition of ROS production could up-regulate intracellular Notch and its downstream effectors (24). These results indicated that Notch might be a downstream target regulated by ROS. Furthermore, we found that cyp1a1, an enzyme involved in drug metabolism, was suppressed by plasma treatment induced Notch inhibition. By promoter analysis we found that there were several classical Notch CSL/RBP-J DNA binding sites (25) in the cyp1a1 promoter region (Supplementary) of either from human species or Mus musculus database, indicating that Notch could directly regulate cyp1a1 expression by modulating cpy1a1 mRNA transcription. Our results reported a new strategy to enhance the drug sensitivity by gas plasma treatment, which might be applied in clinical therapy to overcome drug resistance and to reduce side effects during chemotherapy.

In a whole, we used a new technology, the gas plasma, which could generate various ROS, to enhance the sensitivity of myeloma cells to bortezomib treatment. We further pointed out that plasma could inhibit cyp1a1 via Notch signaling and contribute to the synergistic effect with bortezomib.

**Acknowledgments**

**Funding:** This work is supported by the National Natural Science Foundation of China (Grant No. 51521065), First Class of China Postdoctoral Science Foundation (2017M610639) and Special Fund of Shaanxi Postdoctoral Science Foundation (2017BSHTDZZ04).

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>Cyp1a1:chr9:57867391 [-2000-299](+) [mouse, Mus musculus]

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