Cell apoptosis induced by atmospheric pressure plasma.

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Abstract: Reports show that atmospheric pressure cold plasma could induce cancer cell apoptosis in several minutes. However, up to now, only little has been known about the mechanism of plasma inducing cancer cell apoptosis. In this paper, an atmospheric pressure plasma plume is used to treat human hepatocellular carcinoma (HepG2) cell. Experimental results show that plasma can modulate the intracellular reactive oxygen species (ROS), and nitric oxide (NO) concentration. The relationship between the cell apoptosis and the concentrations of ROS, and NO is analysis. The results indicate that, firstly, the plasma generates NO, which increase the NO concentration of the cells outer environment. Secondly, the intracellular NO concentration is increased due to the NO diffusion from the medium. Thirdly, the intracellular ROS concentration is increased due to the increase of the intracellular NO concentration. Finally, the increase of the intracellular NO, and ROS concentrations result in the HepG2 cell apoptosis.

Keywords: cell apoptosis, atmospheric pressure plasma, NO, ROS

1. Introduction

Apoptosis, also termed programmed cell death, is a kind of self-regulated cell death which can be triggered by a variety of extrinsic and intrinsic signals. In contrast to necrosis, which is caused by acute cellular injury and typified by rapid cell swelling and lysis, apoptosis is morphologically characterized by cytoskeleton disruption, cell shrinkage, membrane blebbing and nuclei fragmentation[1]. The apoptotic program is executed by the caspase family of cysteine proteases acting via a cascade of activation and by nucleases. Apoptosis processes are under the tight control of a number of pro- and anti-apoptotic molecules[2].

As we know plasma can generate nitric oxide (NO), reactive oxygen species (ROS), and so on. On the other hand, cells can also produce NO, a short-lived radical gas, which acts as an intercellular messenger in most mammalian organs, participating in vascular homeostasis, neurotransmission, antimicrobial defense[3], immune system regulation and host defense. It can cause apoptosis, necrosis or protect cells from death, depending on the cell type and the concentration, duration, and site of NO production.

In addition, cells can produce ROS too, which plays a critical role in cancer cell apoptosis, can induce damage to lipids, proteins, DNA molecules and cell death. Intracellular ROS is generated by highly respiring mitochondria and peroxides. Excessive production of reactive oxygen species, such as superoxide anion, hydroxyl radical and hydrogen peroxide, may either directly damage the cellular macromolecule to cause cell necrosis or indirectly affect normal cellular signaling pathways and gene regulation to induce apoptosis. Many stimuli such as tumor necrosis factor, anticancer drugs, and chemopreventive agents stimulate cells to produce ROS and subsequent activation of the cell apoptosis.

Since NO and ROS play important role in inducing cancer cell apoptosis, and they can be produced either by cells themselves or by plasmas. So the NO and ROS generated by cold plasmas may affect the concentration of NO and ROS inside cells when the cells are treated by cold plasmas.

Our previous studies show that plasma treatment can increase the percentage of apoptotic cells, which is associated with cell cycle arrest at the G2/M phase by modulating the genes related to cell cycle and apoptosis. In this paper, to further understand the mechanism of plasma induce cell apoptosis, investigations on the intracellular reactive oxygen species (ROS), and nitric oxide (NO) production concentration after the plasma treatment are carried out. The results indicate that the increase of NO, and ROS production of the HepG2 cell by plasma treatment are involved in HepG2 cell apoptosis.

1. Experimental Arrangement

1.1 Cell line and Cell culture

Human hepatocellular carcinoma cell (HepG2) was purchased from CCTCC (China Center for Type Culture Collection, Wuhan, China). HepG2 cells are maintained in high glucose Dulbecco’s Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY), supplemented with 10% (v/v) fetal calf serum (FCS) (SiJi Qing,
Hangzhou, China) at 37°C in a humidified atmosphere containing 5% (v/v) CO₂.

1.2 In vitro plasma treatment device

A single electrode plasma jet device is used to generate the nonequilibrium plasma plume. The high voltage (HV) wire electrode, which is made of a copper wire with a diameter of 2 mm, is inserted into a 4 cm long quartz tube with one end closed. The inner and outer diameters of the quartz tube are 2 mm and 4 mm, respectively. The quartz tube along with the HV electrode is inserted into a hollow barrel of a syringe. The diameter of the hollow barrel is about 6 mm and the diameter of the syringe nozzle is about 1.2 mm. The distance between the tip of the HV electrode and the nozzle is 1 cm. When helium with a flow rate of 2 l/min is injected into the hollow barrel and the HV pulsed DC voltage is applied to the HV electrodes, a homogeneous plasma is generated in front of the end of the quartz tube, along the nozzle, and in the surrounding air. The length of the plasma plume can be adjusted by the gas flow rate and the applied voltage (amplitude, frequency and pulse width). Detail description of the experimental setup can be found in Ref. 4. The schematic of the device is illustrated in Fig. 1. For all the experiments reported in this paper, the pulse frequency of 8 kHz, pulse width \( t_{pw} \) of 1.6 µs, and applied voltage \( V \) of 8 kV are fixed.

Before the plasma treatment, the medium was changed to 1 mm of phosphate-buffered saline (PBS). Each well of 6-well cluster dishes was placed right under the nozzle during the treatment. The distance between the nozzle and the surface of the PBS was fixed to 10 mm. The working gas He/O₂ (1%) with a flow rate of 1 L/min was used. The experiment was carried out at room temperature. Immediately after treatment, the PBS of the dishes and returned to the CO₂ incubator. The control wells was removed, fresh culture medium was added to each well and incubated in CO₂ incubator for 4h. After the plasma treatment, cells were continuously cultured for 24 h. Then 20 μl of MTT dissolved in PBS solution at a concentration of 5mg/ml was added to each well and incubated in CO₂ incubator for 4h. Finally, the medium was aspirated from each well and 100 μl of dimethyl-sulphoxide (DMSO) was added to dissolve formazan crystals. The optical density of each well was obtained using a microplate Reader (sunrise, Tecan) at 492 nm.

1.4 Intracellular nitric oxide (NO) measurements

HepG2 cells are grown at a concentration of 2.5×10⁴ cells per well and allowed to attach for 8-12 h. After plasma treatment, cells were continuously cultured for 24h. Then cells were washed twice with ice-cold PBS after removal of the media, then lysed using cell lysis buffer (Beyotime, Jiangsu, China). The lysates were collected and centrifuged at 10,000 ×g at 4°C for 10 min. The production of NO was measured by assaying NO₂⁻ in the supernatants using colorimetric Griess reaction. The Griess Reagent System is based on a diazotization reaction which detects the presence of organic nitrite compounds. Nitrite is detected and analyzed by formation of a red pink colour upon treatment of a NO₂⁻-containing sample with the Griess reagent. The optical density of the red pink colour solution could reflect the concentration of the NO₂⁻. The nitrite detection kit was used according to instructions provided by the manufacturer (Beyotime, Jiangsu, China). 50 μl of samples or standard NaNO₂ were incubated with an equal volume of Griess reagent in a 96-well plate at room temperature for 10 min, and the optical density was measured spectrophotometrically at 560 nm (sunrise, Tecan). Absorbance measurements were then converted to moles of NO₂⁻ per well using a standard curve of NaNO₂.

1.5 Detection of intracellular (ROS)

The determination of reactive oxidant species (ROS) level was based on the oxidation of 2,7-dichlorodihydrofluorescin (DCFH) (Beyotime, Jiangsu, China) and performed following the manufacturer’s protocol. 2,7-dichlorofluorescein diacetate (DCFH-DA) is a well-established compound to detect and quantify intracellular ROS production. DCFH-DA can be transported across the cell membrane and deacetylated by esterases to form the non-fluorescent 2',7'-dichlorofluorescein (DCFH). This compound is trapped inside of the cells. Then, DCFH is converted to the highly fluorescent compound DCF through the action of ROS,
which can be detected and quantified by the fluorescence intensity. In brief, following incubation for predetermined times after plasma treatment, cells were collected and washed with DMEM without FBS, and then incubated with 2,7-dichlorofluorescein diacetate (DCFH-DA) at 37°C for 30 min. Dichlorofluorescein (DCF) fluorescence distribution was detected by fluorospectrophotometer analysis at an excitation wavelength of 488 nm and an emission wavelength of 525 nm (PerkinElmer LS55).

2. Experimental Results

2.1 Inhibition of the proliferation of HepG2 cells by plasma

The anti-proliferation effect of plasma on HepG2 cells was shown in Fig. 2. The data indicated the dose dependent decreases in percentage of cell number compared to the non-treated control for each HepG2 cell as the dose of plasma increased from 5 s to 640 s. The estimated dose required to inhibit cell growth by 50% (EC50) was 34.75 s.

Fig. 2. Effect of plasma on the viability of HepG2 cells. Cell viability was determined by MTT assay and was expressed as means ± SD of three separate experiments.

2.2 Increase of intracellular Nitric Oxide by plasma

After treated by the plasma, the concentration of intracellular NO of HepG2 cells is shown in Fig. 3. When the time of plasma treatment was varied from 120 s to 960 s, the concentration of intracellular NO was markedly increased. It is interesting to point out that the intracellular NO production accumulation peaked at 720 s of plasma treatment. This is probably because, with longer treatment time, significant amount of cells appear dead. But we detect the intracellular NO concentration. For dead cells, the NO could not be detected. Thus the average intracellular NO concentration starts to decrease with further treatment.

Fig. 3. Effects of plasma treatment on intracellular NO concentration in HepG2 cells after cultured for 24 hours. The results were expressed as means ± SD of three separate experiments.

2.3 Elevated intracellular ROS induced by plasma

The cells were stained with DCFH-DA to examine the concentration of intracellular ROS in HepG2 cells and the DCF fluorescence was measured by fluorospectrophotometer. As shown in Fig. 4, the level of ROS in cells treated with plasma was increased with treatment time. The intracellular ROS peaks at 720 s too. This is probably due to the same reason as that of the NO mentioned above. These data demonstrated that the plasma treatment significantly increased intracellular ROS production in HepG2 cells, which may promote mitochondrial dysfunction and trigger mitochondria mediated apoptosis.

Fig. 4. Effects of plasma on intracellular reactive oxygen species (ROS) concentration in HepG2 cells after cultured for 24 hours. Results were expressed as means ± SD of three separate experiments.

3. Discussions and Conclusions

As pointed out before, the NO and ROS discussed above can be produced by cells themselves or by plasmas. For the plasma plume that is used to treat the HepG2 cells, although He/O2 (1%) is used as working gas, due to the diffusion of surrounding air, it is suspected that the plasma plume should be able to generate some NO and ROS too.

Certainly, the NO and ROS generated by the plasma could affect the NO and ROS concentrations of the outer medium and the intracellular NO and ROS concentrations when the cells are treated by the cold plasma. Fortunately, the method that we use to measure the intracellular NO can also be used to measure the NO in the cells outer medium. But the ROS concentration in the outer medium could not be measured by using the same method because cells are involved in the method used for the intracellular ROS concentration. Therefore, in the next, in order to tell whether the NO and ROS were generated by the plasma or the cells themselves, firstly, immediately measurements on the intracellular NO were carried out. All the procedures are the same as above for the immediate measurements except without culture for 24 hours. Figure 5 shows the intracellular cell NO concentration immediately after the plasma treatment, which shows the intracellular NO concentration immediately after the plasma treatment is several time higher than that after cultured for 24 hours. Because it takes time for the cells to produce NO, these results imply that the NO is probably mainly produced by the plasma rather than the cells. Figure 5 shows that the NO concentration reaches highest value at 960 s rather than 720 s. This imply that no significant amount of cells die
immediately after the treatment. In other word, it takes time for the cells to die after the plasma treatment.

Secondly, immediately measurements on the intracellular ROS were carried out. Figure 6 shows the effects of plasma on intracellular reactive oxygen species (ROS) generation in HepG2 cells immediately after plasma treatment. As can be seen from Fig. 6, there is no obvious change of the DCF fluorescence intensity after plasma treatment, which is different from Fig. 6. These results suggest that the ROS is probably generated by the cells rather than by the plasma since the ROS generated by cells has 24 hours delay.

Finally, after the plasma treatment, PBS was added to make the total medium equal to 1 mm. Then, immediately measurements on the NO concentration in the medium were carried out following the similar procedures discussed above. Figure 7 shows that the NO concentration in the medium is about five time higher than the intracellular NO concentration immediately after the plasma treatment, which indicates that the intracellular NO is probably diffused from the medium.

In conclusion, the present study is the first to evaluate the plasma dose required to inhibit cell growth by 50% (EC50). The half maximal effective concentration (EC50) refers to the concentration of a drug or toxicant which induces a response halfway between the baseline and maximum after some specified exposure time. It is commonly used as a measure of drug's potency. This data is useful for the plasma in clinical application in the future.

Reactive oxygen species (ROS) is important signal mediators that regulate programmed cell death. ROS are broadly defined as oxygen-containing chemical species with reactive chemical properties. ROS produced by a cell when stimulated by environmental stress or other stimulant is considered as a potential signaling molecule. The increase of ROS stress in cells may cause DNA damage, genetic instability, cellular injury and triggers cell death. Our results show that the treatment of plasma on cells can increase the intracellular ROS concentration.

It is also well known that nitrate tolerance is closely related to oxidative stress of ROS. NO is a pleiotropic mediator and signaling molecule involved in a growing number of cell functions. In some situations, NO activated the transduction pathways causing the cells to undergo apoptosis, whereas in other cases NO was found to protect cells against spontaneous or induced apoptosis. In this study, we found that the intracellular NO concentration increased significantly immediately after the plasma treatment. But the intracellular ROS concentration does not. Therefore we may conclude as follows: firstly, the plasma increases the NO concentration of the cells outer environment. Secondly, the intracellular NO concentration increases due to diffusion. Thirdly, the intracellular ROS concentration increases since the nitrate tolerance of cells is closely related to oxidative stress of ROS as mentioned above. Finally, the increase of the intracellular ROS and NO concentration results the cell apoptosis.

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References