Induction of apoptosis in human breast cancer cells by a pulsed atmospheric pressure plasma jet

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By using an atmospheric pressure plasma jet driven by pulsed dc voltage with repetition rate of several tens of kilohertz, we were able to induce apoptosis in cultured human breast cancer cells (MCF-7). The apoptotic changes in cells with plasma treatment were detected by flow cytometry and fluorescence staining assay. A significant portion of these cells was observed to exhibit the apoptotic fragmentation. Helium plasma with additive O_2 gas was found to be effective in the induction of apoptosis. This plasma jet provides an effective mode of human breast cancer cell therapy. © *2010 American Institute of Physics*. [doi:10.1063/1.3462293]

The apoptotic mode of cell death is an active and encoded process which plays an important role in the regulation and maintenance of individual cells, tissues, or whole organs.[1] Cancer cells can be destroyed with chemicals or radiation but treatments that kill cancer cells are typically toxic to healthy cells as well.[2] In recent years, nonthermal plasma and their applications in biomedical treatments have become hot issues.[3–9] Especially, it has been shown by several groups that plasma is able to induce apoptosis.[3–5] It was shown that plasma acts on the cells directly and not by poisoning the solution surrounding cells, even through a layer of such solution.[3,4] Atmospheric pressure plasma jets are among the most plausible candidates able to destroy cancer cells without damage of healthy cells. Apoptosis can be detected in many ways as follows: by visual observation of the cell shape, by staining of the cell's DNA with propidium iodide (PI) followed by microscopic detection of condensed DNA pieces, by staining externalized phosphatidyl serine (Annexin V assay), etc.[3] Utilizing these methods, this letter aims to elucidate plasma-cell interactions by which nonthermal atmospheric pressure discharge in direct contact with cells is able to induce apoptosis.

Figure 1 shows the jet source. At the center of the glass tube is a tungsten wire with a diameter of 0.6 mm and a pencil-shaped tapered end. The wire is concentric with a glass tube (inside diameter of 10 mm and outside diameter of 12 mm). The glass tube is covered with a stainless steel holder. Several tens of kilohertz pulsed dc high voltage (FT-Lab PDS 4000) is applied to the tungsten wire. The wire shaft is covered with a cone-type Teflon layer tube, leaving a length of 10 mm of the wire exposed to gas. The diameter of the glass tube nozzle is 2 mm. The tip-to-nozzle distance is 10 mm. The glass tube is filled with the gas fed through the six holes (1.5 mm diameter) in the Teflon tube. The gas was delivered at a flow rate in the range of 0.1–1 l/min. As shown in previous study,[10] the plasma jet device exhibits plasma stability while maintaining efficient reaction chemistry and room temperature. The samples are placed on a movable substrate holder with the XY stage. The cells were inoculated punctually and can be treated for a defined time with regulated number of the exposure points.

The interaction of the plasma jet with a living tissue was examined on human breast cancer cells. Human breast cancer cell line (MCF-7) was propagated in Dulbecco's modified Eagles with 10% fetal bovine serum and 100 U/ml penicillin. Cells were incubated at 37 °C with humidified air and 5% CO_2. Then cells were plated in Lab-Tek chamber slides (Nunc™) and small bioassay dishes (Corning) at a density range of 0.5×10^6–3×10^6 cells per chamber, incubated overnight, and exposed to the plasma plume for 5–30 s. Prior to plasma treatment, media from each chamber was almost removed and a small amount of media (a few hundred microliters) was left to keep cells wet during treatment. The distance from the nozzle to the cell surface was 10 mm. The plasma plume reached the cell directly. To verify the apoptosis induction of cancer cells, staining assay and flow cytometric analysis were performed.

Figure 2 represents the fluorescence image of cells among treated and untreated populations. Apoptotic cells can be recognized by stereotypical morphological changes, including membrane blebbing, nuclear and cytoplasmic shrink-

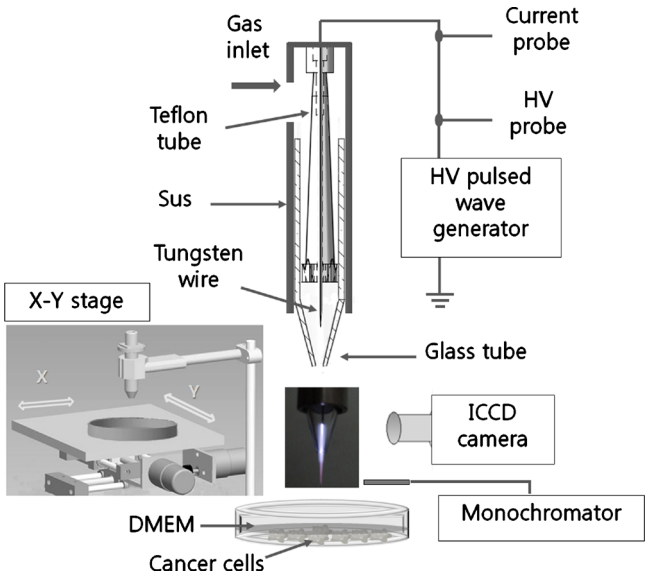

FIG. 1. (Color online) Schematic of the experimental setup.

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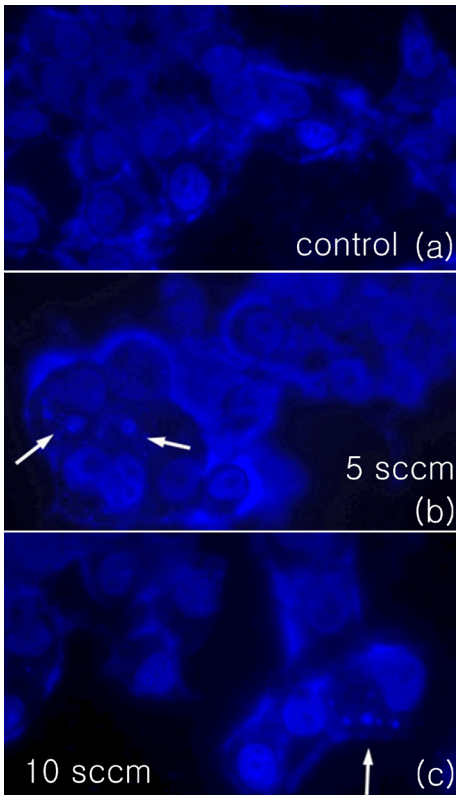

FIG. 2. (Color online) MCF-7 cells were stained with DAPI, and observed under fluorescence microscopy at a magnificence of 400×; arrows indicate apoptotic cells. The sample images observed in the pulsed Ar plasma jet with following different conditions: (a) nontreated control, (b) Ar with the 5 SCCM of oxygen gas, and (c) Ar with the 10 SCCM of oxygen gas.

age, and chromatin condensation. The cells finally are fragmented into compact membrane-enclosed structures, called "apoptotic bodies" which contain cytosol, the condensed chromatin, and organelles.[11] To confirm that the nuclei undergo morphological changes, the cells were exposed to plasma plume for 30 s on four points per Lab-Tek chamber and then cultured for 2 days. The applied voltage, excitation frequency, duty cycle, and gas flow rate were 1.7 kV, 50 kHz, 10%, and 0.1 l/min, respectively. By keeping the gas flow rate small, the desiccation of sample which leads to shrinkage of the cells and to cell death by necrosis[3] was reduced. Since cell growth slows down significantly, the number of dead cells increases in 24 h after treatment.[4] At 48 h after treatment, the cells were fixed in 4% formaldehyde, after which they were incubated in DAPI (4′, 6-diamidino-2-phenylindole) solution (VECTASHIELD H-1200) and nuclear morphology was detected by fluorescence microscopy (Carl Zeiss Axiophot). The images of the samples were obtained in the pulsed Ar plasma jet with different following conditions: (a) nontreated control, (b) Ar with the 5 SCCM (SCCM denotes cubic centimeter per minute at STP) of oxygen gas, and (c) Ar with the 10 SCCM of oxygen gas. Cells display distinctive morphology during the apoptotic process. The end stages of apoptosis are distinguished by the appearance of membrane blebs process. Small vesicles called apoptotic bodies are also observed (arrows). The cell falls apart in several membrane-bound apoptotic bodies and a significant portion of plasma treated cells is observed to exhibit the apoptotic fragmentation.

Figure 3 shows the flow cytometric analysis using Annexin V and PI staining. Apoptosis was assessed by measuring membrane redistribution of phosphatidylserine (PS) using an Annexin V-Fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences 556547). Cells were cultured in 60 mm dish overnight and exposed to the He plasma plume for 10 s on 6 points per dish and then incubated for 2 days. The applied voltage, excitation frequency, duty cycle, and gas flow rate were 1.05 kV, 50 kHz, 10%, and 0.1 l/min, respectively. According to the manufacturer's protocol, cells were harvested, washed with PBS and resuspended in 1×binding buffer containing Annexin V and PI. After incubation for 20 min at 25 °C in the dark, fluorescence-activated cells were detected using a four color flow cytometric analyzer (Beckman Coulter Epics XL), and the data were analyzed using EXPO32 flow cytometry software. In apoptotic cells, the loss of plasma membrane is one of the earliest features. Since externalization of PS occurs in the earlier stages of apoptosis, Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear change. The upper figures represent apoptotic stages in cancer cells induced by plasma exposure. The quadrant I, II, III, and IV denote necrotic, late apoptotic, viable (live), and early apoptotic regions, respectively. When apoptosis is measured over time, cells can be often tracked from both Annexin V and PI negative (viable, or no measurable apoptosis), to Annexin V positive and PI negative (early apoptosis, membrane integrity is present) and finally to both Annexin V and PI

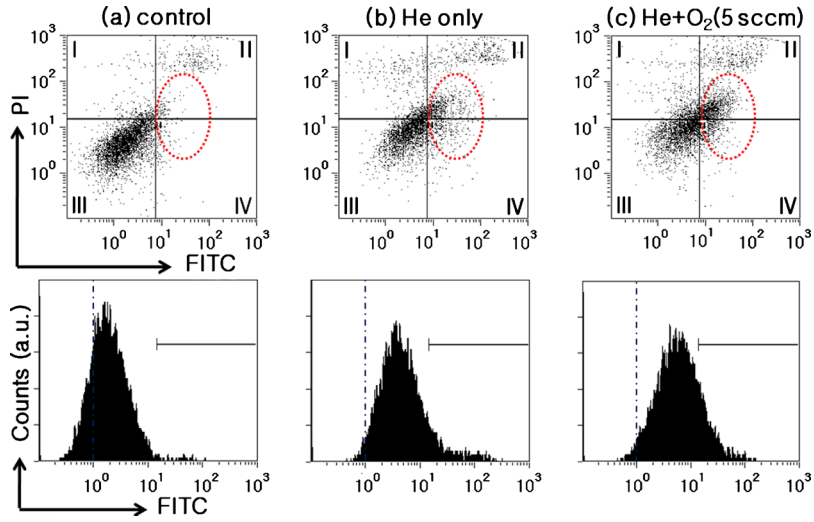

FIG. 3. (Color online) Flow cytometric analysis using Annexin V and PI staining. The apoptosis stages (the upper figures) and the FITC fluorescence histogram (the bottom figures). The analysis was obtained in the He plasma jet with following different conditions: (a) nontreated control, (b) He only, and (c) He with the 5 SCCM of oxygen gas.

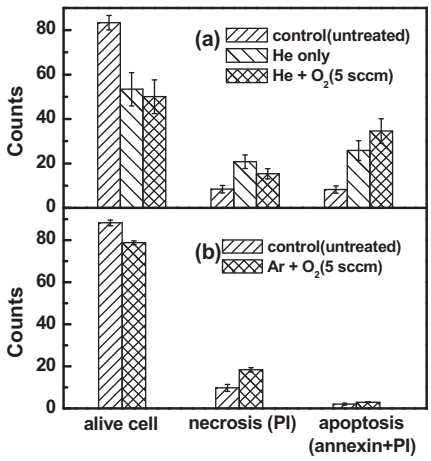

FIG. 4. The increase rates in apoptosis and necrosis of cells after the exposure to plasma. With a flow cytometric analysis, the results after treatment of (a) the He plasma demonstrated a larger apoptotic rate than that of (b) the Ar plasma.

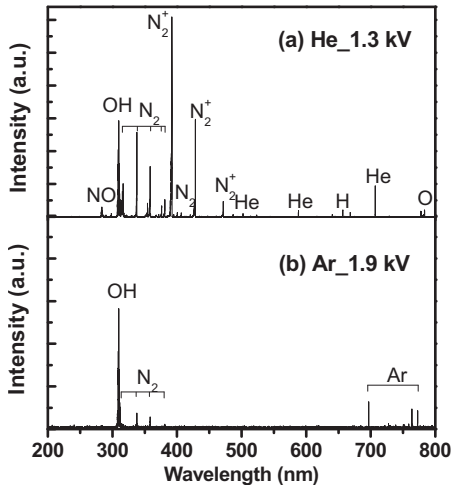

FIG. 5. Optical emission spectra from 200 to 800 nm observed in (a) He and (b) Ar plasma jets.

positive (end stage apoptosis and death). The movement of cells through these three stages confirms apoptosis.[12] The lower figures represent the FITC fluorescence histogram (number of events over fluorescence intensity). Each curve is a histogram of measurements on 10^4 individual cells. Fluorescence intensity was low for untreated cells and increased when cells were exposed on the plasma plume. The flow cytometric images were obtained in the He plasma jet with following different conditions: (a) nontreated control, (b) He only, and (c) He with the 5 SCCM of oxygen gas. The movement to the stages of the early and late apoptosis was identified and the number of expressed cell at those stages (represented by the dotted red circle) was increased in plasma-exposed cells. The comparison of (a)–(c) indicates that the apoptosis of cancer cells is induced by plasma effect and is increased with the addition of a small amount of O_2 gas to pure helium.

Increases in apoptosis and necrosis of cells after exposure to plasma are shown in Fig. 4. The percentage of cell number was analyzed by flow cytometry. With an apoptosis assay, cancer cells after treatment of He plasma demonstrated a larger apoptotic rate than that of Ar plasma. Figure 4(a) represents the ratio of cell numbers for both the helium plasma and the helium-oxygen plasma. Compared to untreated cells, the induction of apoptosis by helium plasma treatment was significantly increased (28% versus 8.9%). Furthermore, the helium-oxygen plasma exposure induced more apoptosis than the pure helium plasma did (38.7% versus 28%). Figure 4(b) shows the percentage of cell numbers exposed to Ar plasma for 30 s on 8 points per dish. The ratio of the apoptosis was lower than that of helium plasma [Fig. 4(a)]. These results reveal that apoptosis frequencies are increased in helium plasma. Furthermore, the addition of oxygen is effective for apoptosis induction.

Figure 5 represents the emission spectra from the plasma jets [(a) He and (b) Ar]. In Fig. 5(a), the strongest emission is the N_2^+ line at 391 nm ($B^2\Sigma_u^+ \rightarrow X^2\Sigma_g^+$), and many nitrogen lines ($C^3\Pi_u \rightarrow B^3\Pi_g$), excited He atom line at 706.5 nm, and excited oxygen line at 777 nm are shown. It should be noted that there appear the emission lines from OH (309 nm), nitrogen oxide (NO) (282 nm), and the H_α line (656 nm). There are many external factors that can induce apoptosis, short-living reactive chemicals such as nitric oxide and reactive oxygen species (ROS), and UV radiation.[8] Therapeutic effects of plasma treatment are caused by chemical reactions with atomic oxygen and nitrogen, hydroxyl radical, and nitrogen oxide rather than by UV and charged ions.[10] As is observed in Fig. 5, the optical spectra of He plasma exhibit an enhanced intensity level of highly reactive radicals such as OH, NO, O, and H compared to the spectra of Ar plasma. This can be accounted for from that metastable He atoms enhance the ionization, excitation, and dissociation of plasma species via Penning effect. One of the mechanisms of apoptosis induction is the DNA damage. The damage can be induced by the attachment of electrons or oxidation by ROS. Since the ROS is in general associated with the induction of cell death, the richness of these species may make the He plasma utilizing a proper ratio of additive O_2 more suitable in cancer therapy.

In summary, a significant portion of human breast cancer cells was observed to exhibit apoptotic fragmentation with the application of a pulsed atmospheric pressure plasma jet. The apoptotic effect depends sensitively on the components of plasma plume.

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