Selective cytotoxic effect of non-thermal micro-DBD plasma

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Introduction

Non-thermal (atmospheric pressure cold) plasma is emerging as a new tool in biomedical applications because it can interact with targeted biomaterials without causing thermal damage to the surrounding Tissue. Recently, the application of non-thermal plasma for cancer therapy has attracted a great deal of attention because plasma has been shown to induce apoptosis in several types of cancer cells including liver cancer, melanoma (G361) cancer, melanoma skin cancer, breast cancer, and brain cancer cells. Non-thermal plasma selectively eradicates cancer cells without serious damage to normal cells. These radicals can be generated by the plasma itself or by interactions between plasma and the surrounding air. In order to establish the use of non-thermal plasma as an alternative cancer treatment, the plasma effect needs to be evaluated under various conditions and the mechanism of apoptosis should be determined. In this work, we focused on the selective effects of non-thermal micro-DBD plasma on different cell types.

Materials and Methods

We used a non-thermal atmospheric pressure micro-DBD plasma device that consisted of two upper parallel silver electrodes with a width of 200μm thickness of 5μm and gap distance of 200μm, silicon dioxide (SiO2) dielectric layer 30μm in thickness, hydration prevention layers of aluminum oxide (Al2O3) 1μm in thickness, and a magnesium oxide (MgO) layer 1μm in thickness. The plasma discharge area was about 35mm in diameter. The non-thermal micro-DBD plasma operates at atmospheric pressure in air when a time-varying voltage waveform is applied to the two electrodes. The discharge energy delivered to the cells during the plasma generation was 5.58×10^{-5} J/cycle. Cells were seeded at a concentration of 1×10^{5} cells/well in a 35mm Petri dish. The cells were 3.5mm away from the plasma source and were exposed to the micro-DBD plasma with nitrogen (N2) (99.99% purity) feed gas.

Two human cervical cancer cell lines, HeLa and SiHa, and HFBs were used in this work. HFBs have been widely used as a control in cervical cancer cell studies, and thus the genetic background of this cell type was used to determine gene expression outcomes in this study. The cells were cultured in a humidified 37 °C incubator with 5% CO2, and the media was changed every other day until cells were approximately 80% confluent.

Cell viability was measured using a cytotoxicity assay kit. The cells were treated with non-thermal DBD plasma for different exposure times ranging from 0 to 8 min. After a 24 h incubation period, EZ-Cytox solution was added to each well at 10μl/well and the plates were incubated at 37 °C for 20 min. Absorbance was read at 450 nm using a spectrophotometric microplate reader. All experiments were performed at least in triplicate.

The significance of the difference between control and plasma-treated groups was assessed via the Student t test. p-values less than 0.05 were regarded as statistically significant.

Results

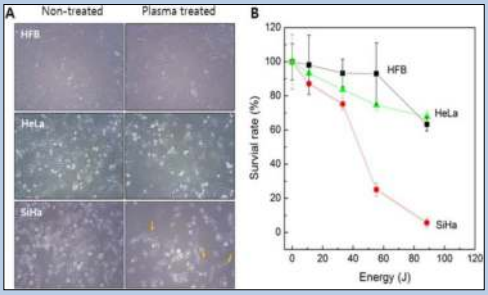

Figure 1.

(A) Morphological changes of cervical cancer cells (HeLa and SiHa lines) and HFBs treated with non-thermal micro-DBD plasma. Cells before and after 3 min plasma treatment photographed under a phase contrast microscope (200×magnification).

(B) MTTassay was performed to evaluate the cytotoxicity of the non-thermal micro-DBD plasma.

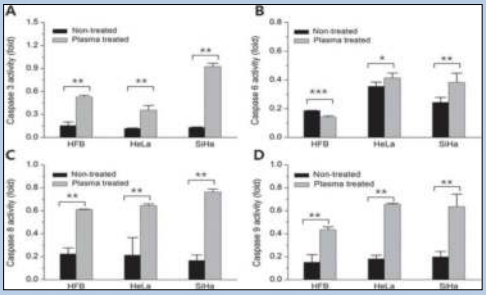

Figure 2.

Effects of non-thermal micro-DBD plasma on caspase activity in cervical cancer cells (HeLa and SiHa lines) and HFBs. The caspase activities induced by the non-thermal micro-DBD plasma were determined by colorimetric assays using caspase-3, -6, -8, and -9 activation kits. The cells were treated with plasma of E=33 J (for 3 min) and the caspase activity were determined 24 h after the treatment. The caspase activity was analyzed after washing with PBS buffer. Each value represents the mean ± SD (*p<0.05, **p<0.01, t-test).

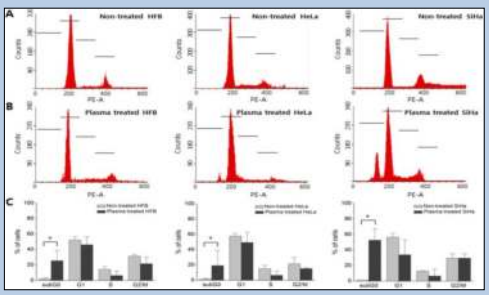

Figure 3.

Cell cycle analysis of cervical cancer cells (HeLa and SiHa lines) and HFBs performed (A) before and (C) after the plasma treatment. The cells were treated with plasma of E=33 J (for 3 min) and the cell cycle were determined 24 h after the treatment.
(B) and (D) The percentages of cells in the subG0, G1, S and G2/M phases of the cell cycle were counted. The cell cycle was analyzed after washing with PBS buffer. Each value represents the mean ± SD (*p<0.05, **p<0.01, ***p<0.001,t-test).

Discussion

Since the demonstration of cellular apoptosis induced by plasma, the use of non-thermal atmospheric plasma has attracted a great deal of attention as a next generation cancer therapy. Moreover, some reports have shown that the effects of plasma are highly selective for cancer cells.

In this work, we also observed that non-thermal micro-DBD plasma produced selective apoptotic effects in cancer cells. As shown in the MTT assay results, micro-DBD plasma inhibited cell proliferation in both cervical cancer cells and normal cells; however, the inhibition was more significant in the cancer cells than normal fibroblasts. Michael et al suggested that cancer cells respond more sensitively to plasma than normal cells because of the greater percentage of cells in the S phase of the cell cycle. In the cell cycle of the normal cells (keratinocytes) and two cancer cell lines (308 and PAM212 cells), cells in S phase constituted 10% of normal cells, but 50% of 308 cells and 45% of PAM212 cells. In this work, we also analyzed the cell cycle in all cells before and after non-thermal micro-DBD plasma treatment. The changes in the cell cycle induced by plasma were clearly observed in all cells. Compared to untreated cells, treated cells showed a significant increase in subG0 cells, indicating that cellular apoptosis was induced by the plasma.

Plasma acts on the cell membrane, resulting in lipid peroxidation, transient pore formation, and alteration of protein structure. The free radicals generated by plasma act as an apoptotic stimulus by involving the mitochondria. A low dose of plasma can stop cancer cell growth and induce apoptosis, while a high dose causes necrosis. Non-thermal micro-DBD plasma treatment inhibits the proliferation of cervical cancer cells through cell cycle arrest and induction of apoptosis.

The most interesting thing observed in this work is that non-thermal micro-DBD plasma induced selective anti-proliferative effects in cervical cancer cells compared to HFBs. Alteration of gene expression profiles by plasma was considerably higher in cervical cancer cell lines than HFBs. The survival rate of SiHa cells was also lower than that of HeLa cells during the entire process (11<E<88 J). To understand the difference in proliferation inhibition between cervical cancer cell lines and between cervical cancer cells and HFBs, we investigated the transcriptome for genes affected by non-thermal DBD plasma. Gene expression profiling revealed that non-thermal DBD plasma significantly altered gene expression in cervical cancer and normal HFB cells. Alteration of gene expression profiles by plasma was considerably higher in cervical cancer cell lines than HFBs. Moreover, plasma caused significantly more alterations of gene expression in SiHa compared with HeLa. The extent of alteration in gene expression profiles induced by plasma was consistent with the differential cytotoxic effects observed in each cell line and that was most likely to be important for the tumor-selective effects of DBD plasma.

We also performed GO and pathway analyses to identify tumor-selective biological effectors among the gene expression profiles altered by DBD plasma. The expressed genes were grouped into 559 clusters based on their specificity. In SiHa cells, the apoptosis related gene cluster, which contained about 80 genes, was highly regulated and ranked fourth in the 559 clusters. We did not observe any DBD plasma mediated transcriptional alteration of apoptosis-related genes in normal or HeLa cells. The apoptosis-related genes were divided into pro-apoptotic genes and anti-apoptotic genes. We detected increased expression of pro-apoptotic genes (CHAC1, RHOB, PMAIP1, TNFAIP8, TNFRSF19 and TNFRSF1, etc) and decreased expression of anti-apoptotic genes (RTKN, AKT1, BCL2L1, TIAF1, NOL3 and BIRC2, etc) in the cluster of apoptosis-related genes. Genes encoding components of the intrinsic and extrinsic apoptotic pathway were selectively induced by the preferential cytotoxic effects of DBD plasma in SiHa cells. In particular, altered gene expression of RTKN and RHOB was closely related to DBD plasma-induced apoptosis. Our microarray assays indicated that non-thermal micro- DBD plasma induced a 7-fold decrease in the expression of RTKN genes and a 2.5-fold increase of RHOB in SiHa cells, which resulted in a dramatic increase in apoptosis in plasma-treated SiHa cells.

DBD plasma also mediated the opposing expression pattern of apoptosis-related genes, leading to decreased expression of pro-apoptotic genes and increased expression of anti-apoptotic genes, which cause tumor cell proliferation and anticancer drug resistance events. Our cytotoxicity and gene expression profile results support the hypothesis that a combination of two distinct mechanisms, differential alteration of global gene expression and specific changes in pro- and anti-apoptotic gene expression, is the basis for the selective cytotoxicity of DBD plasma in cervical cancer cells.

Conclusion

In summary, non-thermal DBD plasma showed selective apoptotic effects depending on cell type. Plasma treatment was more effective in cervical cancer cells compared to HFBs. In cervical cancer cells, the effects of plasma on SiHa cells were stronger than its effects on HeLa cells. This evidence of selective apoptosis was in agreement with the observed gene expression alterations induced by plasma treatment.