Low Dose Non-Thermal Plasma Interacts with Mammalian Cells Indirectly through Modification of the Cell Culture Medium

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Abstract: Non-thermal plasma is now being widely considered for various medical applications. However, understanding of interaction between plasma and cells is lacking. We investigated the possibility that effects of the non-thermal plasma treatment can penetrate through cell membranes. The results indicate that plasma treatment at low power produces DNA double strand breaks in mammalian cells, suggesting plasma penetrates the cells. Plasma interacts with cells indirectly by producing reactive oxygen species (ROS) in the cell medium.

Keywords: Non-Thermal Plasma, Plasma Medicine, Dielectric Barrier Discharge, DNA

1. Introduction

Thermal plasma has been employed in medicine for coagulation and ablation for some time [1]. Treatment of tissues and cells by non-thermal plasma, where the gas temperature is nearly at room temperature, is a recent development [2]. It has been noted that non-thermal plasma applied directly to surfaces of living tissues can coagulate blood; however, it does so without charring the tissue [2]. Similarly, non-thermal plasma appears to kill bacteria on the surface of living tissue without histologically visible damage [2]. It has been reported that non-thermal plasma can also mediate attachment of cells to substrates [3-5], increase transfection efficiency [6, 7]. Ability to tune non-thermal plasma effects together with the simplicity of plasma generating devices and localized nature of plasma application makes it a promising tool in medicine. However, mechanisms of interaction between non-thermal plasma and living systems have been poorly understood.

Here we study the mechanisms of interaction between non-thermal plasma and mammalian cells. Several different methods of non-thermal plasma generation at atmospheric pressure are known. The type of non-thermal plasma employed in this study is called the Dielectric Barrier Discharge (DBD). It was invented by Siemens in 1859 [8]. The plasma in this discharge is created when the time-varying high voltage reaches sufficient magnitude to cause air breakdown. The presence of dielectric layer (dielectric barrier) in the path of the discharge limits its current which, in turn, limits the energy transferred to ions and neutral gas species keeping their temperature low. Although the plasma temperature is low, the presence of charged particles, radicals and electronically excited molecules and atoms makes DBD plasma a potentially active medium whose exact properties can be controlled to some extent through gas composition as well as waveform of the time-varying applied voltage.

2. Materials and Methods

Cell Culture

Mammalian Breast Epithelial Cells (MCF10A) were maintained in high glucose Dulbecco's Modified Eagle's Medium-Ham's F12 50:50 mixture (DMEM-Ham's F12 50:50) (Cellgro, Mediatech, VA, USA) supplemented with 5% donor horse serum (Sigma Aldrich, St. Louis, MO, USA), Epidermal Growth Factor (EGF, 100 µg/ml, Sigma Aldrich, St. Louis, MO, USA), Hydrocortisone (1 mg/ml, Sigma Aldrich, St. Louis, MO, USA), Cholera Toxin (1 mg/ml, Sigma Aldrich, St. Louis, MO, USA), Insulin (10 mg/ml, Sigma Aldrich, St. Louis, MO, USA) and Penicillin/Streptomycin (500 □l, 10000 U/ml penicillin and 10 mg/ml streptomycin, Sigma Aldrich, St. Louis, MO, USA). Media was changed every two days. For plasma treatment, cells were washed with phosphate buffered saline (PBS), detached with 0.25% trypsin (GIBCO, Invitrogen, CA, USA), and seeded near confluence (4 x 10^5 cells/well) on 22 x 22 mm square glass cover slips (VWR, PA, USA) in 6-well plates (Greiner Bio One, NC, USA). Cells were cultured for 24 hours prior to plasma treatment in 2.0 ml supplemented media in a 37°C, 5% CO_2 incubator to allow full attachment and spreading.

Amino Acids; Serine, Methionine, Cysteine, Arginine Leucine, Lysine, Isoleucine, Valine, Proline, Glutamic Acid, Glutamine, (100 µM, Sigma-Aldrich, St Louis, MO) were used to treat cells directly and separately to determine their role in inducing DNA damage after plasma treatment.

N-Acetyl-L-cysteine (2 mM, Sigma-Aldrich, St Louis, MO), an intracellular reactive oxygen species

(ROS) scavenger and sodium pyruvate (10 mM, Sigma-Aldrich, St Louis, MO), an extracellular ROS scavenger were used to block the reactive oxygen species produced by non-thermal plasma treatment.

Plasma Treatment

Non-thermal atmospheric pressure dielectric barrier discharge plasma was produced using an experimental setup similar to one previously described and schematically illustrated in Figure 1 B. The non-thermal plasma was generated by applying alternating polarity pulsed (500 Hz – 1.5 kHz) voltage of ~20 kV magnitude (peak to peak), 1.65 µs pulse width and a rise time of 5 V/ns between the insulated high voltage electrode and the sample undergoing treatment using a variable voltage and variable frequency power supply (Quinta, Russia). 1 mm thick, polished clear fused quartz was used as an insulating dielectric barrier covering the 1 inch diameter copper electrode. The discharge gap between the bottom of the quartz and the treated sample surface was fixed at 2 mm. Discharge power density was measured to be 0.13 Watts/cm^2 (at 500Hz) and 0.31 Watts/cm^2 (at 1.5 kHz) using both electrical characterization and a specially designed calorimetric system.

As shown in Fig. 1 MCF10A cells on glass cover slips were exposed to non-thermal plasma at various doses from 0.13 J/cm^2 to 7.8 J/cm^2. Briefly, each cover slip was removed from the 6-well plate and placed on a microscope slide, which was then positioned on the grounded base of the plasma device. 100 µl of supplemented media was added to the glass cover slip before plasma treatment to prevent sample drying. Following plasma treatment, the cells were held in the treated medium for one minute and then the cover slip was placed in a new 6-well plate, 2 ml of supplemented media was added to the well, and the samples were returned to the incubator for one hour before analyzing the samples using immunofluorescence or western blot.

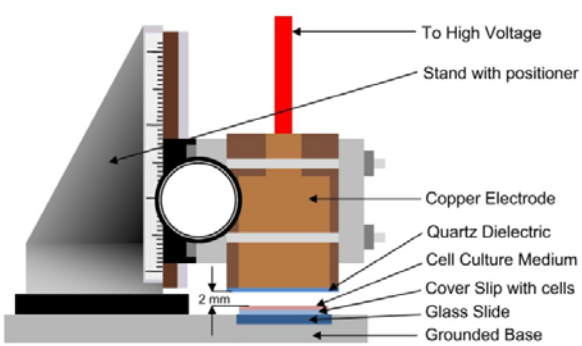

Fig. 1 Plasma Treatment Setup

Three different approaches were used for non-thermal plasma-treatment of cells *in vitro*: direct, indirect and separated. In direct treatment, the sample itself was one of the electrodes that created the plasma discharge, as illustrated in. Plasma discharge occurred between the powered high voltage electrode quartz surface and the sample surface, which exposed the sample directly to both neutral reactive species and charged particles. In contrast, for indirect treatment, a grounded mesh was placed between the high voltage electrode and the treated sample to prevent charged particles from reaching the sample surface. In separated plasma treatment, medium alone was plasma treated separately from cells and then immediately applied to cells. In this case, cells were not in direct contact with any plasma component.

Western Blot

Protein expression and modification were analyzed by immunoblot. Total cell lysates were prepared by direct lysis of washed cells in 2X SDS sample buffer containing β-mercaptoethanol. Samples were electrophoresed at 150 V in Tris-glycine SDS running buffer [25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS (pH 8.3)]. Following electrophoresis, proteins were transferred on to PVDF (Millipore, MA, USA) membrane for two hours in Tris-glycine transfer buffer [10% SDS, Deionized Water, Tris-Glycine and Methanol (VWR, PA USA)]. Immunoblotting was done by blocking membranes in 1% nonfat dried milk (Carnation) in PBS with 0.1% Tween 20 (PBST) for α-tubulin or 5% bovine serum albumin (BSA, Fraction V, Fisher Scientific) in PBST for g-H2AX followed by incubation with primary antibodies in 1% nonfat dried milk in PBST for a-tubulin and 5% BSA in PBST for γ-H2AX overnight for 10 to 12 h at 4ºC with rocking. Primary antibodies used for immunoblot included mouse monoclonal antibodies specific for γ-H2AX [phospho-histone H2AX (serine 139), clone JBW301; Upstate] and α-tubulin (Santa Cruz Biotechnology). The primary antibodies were detected with fluorescently tagged goat anti-mouse Alexa and Fluor 488 (Santa Cruz Biotechnology). Immunoblot was developed using Odyssey Infrared Gel Imaging system (LI-COR Biosciences, NE, USA).

Colony Survival Assay

4 x 10^5 MCF10A cells were seeded on 22 x 22 mm square cover slips in 6-well plates one day before plasma treatment. Cells were plasma treated as described at various doses of plasma and then incubated for one day after plasma treatment. 300 cells were seeded onto 60-mm dishes after exposure to non-thermal plasma or H_2O_2 (positive control). Colonies, which formed 11 days after plating MCF10A cells, were fixed and stained with a crystal violet solution (0.5% in 20% ethanol) and then counted. Assays were done in triplicate.

3. Results

To determine whether DBD plasma treatment of cells induced DNA damage, we looked at phosphorylation of H2AX, a histone variant that is phosphorylated in

response to DNA double strand breaks [9]. Western blot with an antibody that detects phosphorylated H2AX (γ-H2AX) revealed that plasma treatment of cells induces a dose-dependent increase in the level of γ-H2AX (Fig. 2).

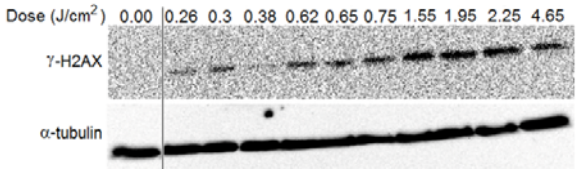

Fig. 2 MCF10A cells were treated with the indicated dose of DBD plasma as described. After one hour incubation, lysates were prepared and resolved by SDS-PAGE and representative immunoblots with antibody to γ-H2AX (upper panel) or α-tubulin (lower panel) are shown

Indirect immunoflorescence revealed foci of γ-H2AX (Fig. 3), which were increased in number at higher doses (data not shown).

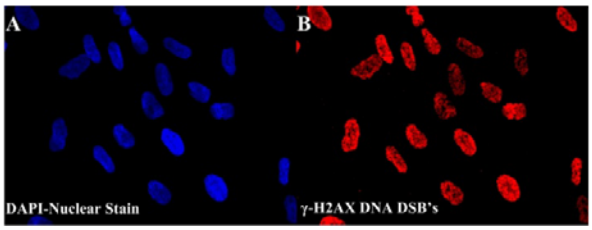

Fig. 3. Indirect immunofluorescence of MCF10A cells one hour after treatment with 1.55 J/cm^2 DBD plasma

Effects on cell survival were determined by measuring colony formation. Cells treated at low doses of plasma (≤ 0.65 J/cm^2) showed no significant decrease in survival, whereas survival decreased with increased dose of plasma (Fig. 4), suggesting that at higher doses DBD plasma may be inducing cell death.

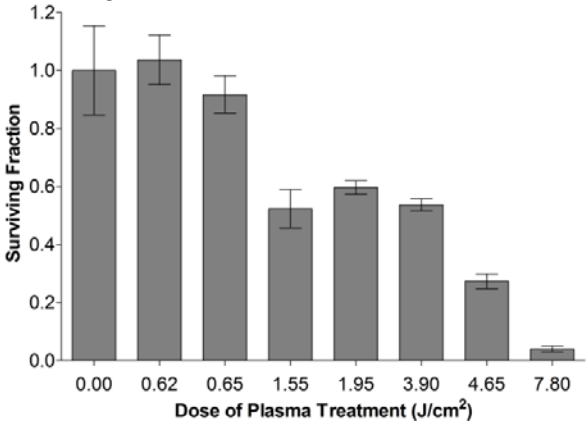

Fig. 4 Cells were treated with the indicated dose of DBD plasma; one hour after treatment, 300 cells were plated in a 6 cm dish and colonies were counted after 8 days. Data from triplicate samples (±S.E.M.) are expressed relative to the # of colonies in the untreated control.

One possible mechanism underlying these effects is generation of reactive oxygen species ROS, which at low levels induces cell proliferation and at high levels induces cell death through DNA damage [10]. DNA damage induced by ionizing radiation has been shown to result from formation of ROS [11, 12]. We next sought to directly test whether the damage induced by DBD plasma is due to ROS (e.g. H_2O_2, OH^-, singlet oxygen, etc.) generated in the media and/or cells by plasma treatment. Cells were pre-treated with the ROS scavenger, N-acetyl cysteine, which was found to block induction of □-H2AX even at high doses of DBD (Fig. 5), suggesting that the effects are mediated by ROS.

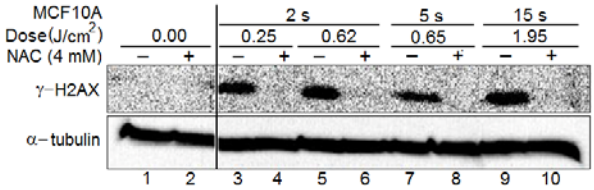

Fig. 5 MCF10A cells were incubated for 2 hours with 4 mM N-acetyl cysteine (NAC) (+) or cell culture medium (-), followed by treatment with the indicated dose of DBD plasma. γ-H2AX (upper panel) or α-tubulin (lower panel) was detected by immunoblot of cell lysates prepared one hour after plasma treatment.

Consistent with this finding, cells held for longer periods of time in the 100 µl of treatment volume before dilution into media accumulated higher levels of damage, and the amount of damage was decreased when the media was diluted immediately after treatment (Fig. 6). These data suggest that ROS may accumulate in the cells during the incubation.

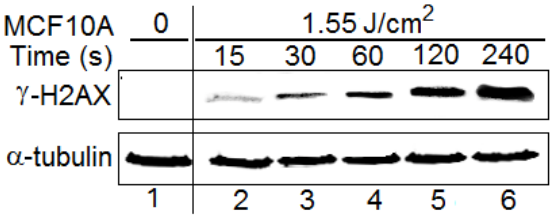

Fig 6. ROS accumulate in media and effects on cells are dependent on their concentration. Cells on cover slips overlaid with 100 µl cell culture media were treated with 1.55 J/cm^2 DBD plasma, followed by dilution in 2 ml of media at the indicated holding time after treatment. Cell lysates were subjected to immunoblot after 1 hour of incubation

Non-thermal plasma is known to produce many charged (electrons and ions) and long living and short living neutral species (metastable particles, OH radical, ozone, singlet oxygen, H_2O_2) in gas phase as well as in liquid phase. To determine which of these species are responsible for the induction of DNA damage, DBD plasma was applied in different modes. Previous experiments have

involved direct treatment, in which all the species produced by plasma come in contact with the surface of cell culture medium covering the cells during treatment. Indirect treatment involves placement of a grounded mesh between the high voltage electrode and the medium covering the cells being treated, which blocks charged species and allows only neutral species to come in contact with the surface of the medium. Comparison of DNA damage by direct plasma treatment and indirect plasma treatment shows that phosphorylation of H2AX was not significantly different in indirect vs. direct treatment (Fig. 7), indicating that active neutral species produced by DBD plasma are responsible for the induction of DNA damage.

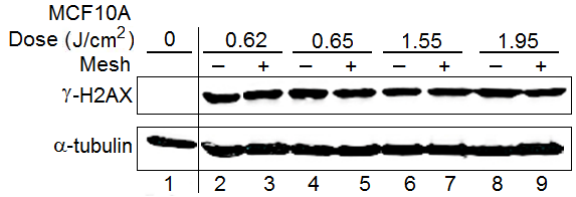

Fig 7. Cells were subjected to DBD as described earlier (direct, -) or a grounded mesh was placed between the electrode and the medium (indirect, +).

To determine whether the effects of DBD plasma are due to modification of the cell medium by plasma treatment, the medium was treated in the same way without cells and then added to cells (separated treatment). As shown in Fig. 8, damage induced by the treatment of the medium separately from cells was not less than that produced by direct treatment. This suggests that ROS generated in the medium by plasma treatment are responsible for the induction of DNA damage. These ROS species must survive long enough to remain active while being transferred to the cells.

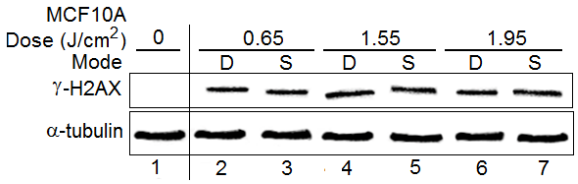

Fig. 8 Cells were subjected to DBD plasma as described earlier (direct, D) or media (100 µl) was subjected to DBD plasma and then transferred to the cells (separate, S).

To determine how long living are these ROS species, cell medium was separately treated as described above and then held for increasing times before being added to cells. Induction of DNA damage by the medium treated with DBD plasma was not significantly reduced by holding media up to one hour prior to adding it to cells (Fig. 9), suggesting that neutral species (as shown above charges do not play a major role) may react with organic components in the cell medium to produce long living organic peroxides which are known to have a half life on the order of 12-24 h [13].

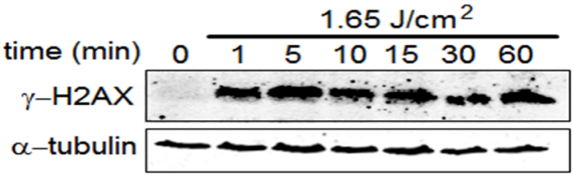

Fig. 9 Media (100 µl separated treatment) was subjected to DBD and was transferred to cells after holding for 1 to 60 min. After 1 minute incubation with cells, cover slips with treated media and cells were transferred to a dish with 2 ml of media.

4. Conclusions

Use of DBD plasma for clinical applications requires an understanding of its interaction with living tissues. Understanding the mechanism underlying the effects of plasma is an essential first step in applying it to clinical use. Low dose non-thermal plasma interacts with mammalian cells indirectly through modification of the cell culture medium. We confirmed that long living reactive oxygen species (ROS) produced by plasma in cell culture medium mediate interaction between plasma and DNA inside mammalian cells. Current studies are directed at establishing which component of the cell culture medium is modified by non-thermal plasma treatment. Future work will involve investigating the mechanism by which long living ROS produced by plasma penetrate the cellular membrane and induce DNA damage.

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