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ORIGINAL PAPER

Floating Electrode Dielectric Barrier Discharge Plasma in Air Promoting Apoptotic Behavior in Melanoma Skin Cancer Cell Lines

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Abstract Initiation of apoptosis, or programmed cell death, is an important issue in cancer treatment as cancer cells frequently have acquired the ability to block apoptosis and thus are more resistant to chemotherapeutic drugs. Targeted and

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perhaps selective destruction of cancerous tissue is desirable for many reasons, ranging from the enhancement of or aid to current medical methods to problems currently lacking a solution, i.e., lung cancer. Demonstrated in this publication is the inactivation (killing) of human Melanoma skin cancer cell lines, in vitro, by Floating Electrode Dielectric Barrier Discharge (FE-DBD) plasma. Not only are these cells shown to be killed immediately by high doses of plasma treatment, but low doses are shown to promote *apoptotic behavior* as detected by TUNEL staining and subsequent flow cytometry. It is shown that plasma acts on the cells directly and not by ''poisoning'' the solution surrounding the cells, even through a layer of such solution. Potential mechanisms of interaction of plasma with cells are discussed and further steps are proposed to develop an understanding of such systems.

Keywords Non-thermal plasma · Dielectric barrier discharges (DBDs) · Apoptosis · Melanoma cancer cells · Cancer treatment · Skin diseases

Introduction

Apoptosis, or programmed cell death, is a complex biochemical process of controlled self-destruction of a cell in a multicellular organism [1, 2]. This process plays an important role in maintaining tissue homeostasis, fetal development, immune cell ''education'', development, and aging. Examples of apoptosis that occur during normal body processes include the formation of the outer layer of skin, the inner mucosal lining of the intestine, and the endometrial lining of the uterus, which is sloughed off during menstruation. During apoptosis, cellular macromolecules are digested into smaller fragments in a controlled fashion, and ultimately the cell collapses into smaller intact fragments that can be removed by phagocytosis without damaging the surrounding cells or causing inflammation. In contrast, during *necrosis*, also termed ''accidental cell death'', the cell bursts and the cellular contents spill out into the extracellular space, which can cause inflammation. Necrosis is induced by cellular injury, for example, extreme changes in osmotic pressure or heat, that lead to adenosine tri-phosphate (ATP) depletion of the cell. With *cancer* cells, however, a problem arises with apoptosis as the tumor cells frequently ''learn'' how to turn off apoptosis as one of the processes they employ in evading the immune system and surviving under unfavorable conditions. For this reason, for example, chemotherapy as means of treatment of breast, colon, and lung cancer met limited success [1]. In general, the employment of systemic chemotherapy drugs to induce apoptosis in cells that try to block it is not an easy task, as these drugs tend to affect all cells in the body [1, 2]. A way to target apoptosis development only in specific areas of the body is needed and a method to do so is offered in this paper where apoptotic behavior is promoted in Melanoma cancer cell lines following low doses of non-thermal plasma treatment insufficient to destroy the cell immediately.

In recent years, non-thermal plasma discharges have been gaining popularity in the materials processing industry for their ability to selectively modify a surface with minimal, if any, damage to this surface and practically no change to the bulk material. This way, for example, a surface of an implant may be made biologically compatible to the tissues and cells it will come in contact with, while the bulk material of the implant can be tailored to desirable mechanical properties like high strength, low weight, durability, fracture resistance, etc. Recently, the demand for

sterilization and disinfection of various surfaces increased and non-thermal plasmas were found to be an effective solution. Many groups worldwide have successfully demonstrated plasma's ability to treat, disinfect, and sterilize various targets. Plasmas are widely used in textile [3–5] and lighting industries [6–8], electronics [8–11], and in many other applications (see [8–12], for example). It is no surprise that biology and medicine also employ the ''fourth state of matter'' in materials processing [13–15], sterilization [10, 16–23], improvement of bio-compatibility [13, 24, 25], tissue engineering [26–28], to increase adhesion and wettability and for other surface modifications [29–34]. Medical applications focused on plasma treatment of living tissue, which are of growing interest these days, require treatments at atmospheric pressure since cells and tissues are not vacuum-compatible [16, 35]. Atmospheric pressure treatments can be separated into two major categories: where *temperature* is used as means of treatment [36–40], and where active species, radicals, or ultraviolet radiation generated by plasma are used for *targeted* chemical modification and catalysis [8, 10, 16, 41–48]. Thermal plasmas are widely used in medicine today both in attached arc mode where arc contacts the tissue directly [40, 49–51] and in a ''jet'' mode where gas (usually argon) is blown through the plasma but remains at high temperature [36, 37, 39, 52]. Both attached arc and thermal jet are known and used for their ability to rapidly coagulate blood [36–40, 50, 52]; however, they can cause significant thermal tissue desiccation, burning, and eschar formation[1]. Additionally, excessive smoke could be a problem during thermal treatment of tissues. During open-air treatments smoke can be removed by means of vacuum suction (though it is not a simple procedure); however, during endoscopic treatments smoke becomes a major issue where it is very difficult to remove and obstructs the view of the camera. For these reasons, development of non-thermal plasma methods of treatment where temperature does not exceed 60°C is needed.

Recently, the focus of the plasma community has shifted to applications where tissue damage and desiccation are minimized or eliminated [35]. Many configurations have been proposed for treatment of biological surfaces, cells, and tissues. The ''plasma needle,'' for example, can possibly be used for selective cell detachment [28, 30, 45]. It involves a corona discharge igniting at the end of a sharp tip in helium upon application of radio frequency (~13 MHz) electromagnetic excitation. This discharge operates at near room temperature, dissipating milliwatts in several cubic millimeters. Suggested applications for this technique include treatment of dental cavities and skin disorders. This plasma has been demonstrated to destroy cells and bacteria in a highly localized fashion without disrupting the nearby tissue [45]. Recently, it was also shown that this plasma promotes inactivation in mouse fibroblast cells, where apoptosis-like behavior is observed after treatment—the cells appear to clump up and die [53]. Another promising use of non-thermal plasmas is reversible pore formation for targeted drug delivery [28, 30] or irreversible pore formation [30, 54]. Pulsed Electron Avalanche Knife (PEAK) is one more plasma-based surgical tool where thermal damage to tissue is reduced by keeping the current pulses short (microseconds) and the electrodes thin (microns). Even though the device operates in the high current regime, timescales of treatment are not enough to heat up the tissue, resulting in non-damaging treatment [55, 56]. PEAK was successfully demonstrated in precise cutting with minimal damage. However, these

[1] In medicine, the term ''eschar'' describes a slough or dry scab that forms on an area of skin that has been burnt or exposed to corrosive agents. The term ''eschar'' is commonly confused with ''char.''

systems are designed for precise treatment or cutting of very small areas and another system, capable of treating large areas of living tissue, is discussed in this paper. The Floating Electrode Dielectric Barrier Discharge (FE-DBD) system, constructed similarly to conventional dielectric barrier discharges (DBDs) is inherently non-thermal—it is able to operate at room temperature and pressure [10, 57]. This system operates at power densities of 0.1–2 W/cm^2. FE-DBD was applied for complete skin sterilization without any damage to skin and blood coagulation without damage to surrounding tissue [16].

Presented in this paper is a way to treat cells where immediate destruction and necrosis is not desired or directly achieved by plasma. FE-DBD plasma treatment is shown to initiate *apoptosis* in Melanoma cancer cell lines—a threshold at which plasma treatment does not cause immediate necrosis but initiates complex cascade of biochemical processes leading to cell death many hours and even days following the treatment. Melanoma cells, treated by plasma at doses significantly below those required for cell destruction, survive the plasma treatment but develop apoptosis many hours post treatment and die (disintegrate) by themselves gracefully. This could potentially be an intriguing new idea for cancer treatment, especially if by manipulation of plasma parameters the treatment could be made selective to cancerous cells over healthy cells, as was demonstrated before for bacteria vs. healthy cells [16].

Following this introduction, Sect. 2 briefly discusses construction of the Floating Electrode DBD system used for the treatment. Sections 3 and 4 present details of the biological preparations and experiments performed on Melanoma cancer cell lines including growing these cells, their life path, treatment dynamics, apoptosis assays, and flow cytometry. Section 5 describes treatment of these cell lines for inactivation and necrosis, while Sect. 6 describes modes where the cells have not been inactivated by plasma but developed apoptosis many hours and days following the plasma treatment. This paper is concluded with ideas for future work to further analyze plasma-induced apoptosis mechanisms and their dependence on specific plasma-generated chemistry.

Floating Electrode Dielectric Barrier Discharge

In our experiments, we apply continuous-wave high voltage (10–30 kV) to a quartz-protected electrode that generates plasma between the quartz and the surface of living tissue of a human or animal, or a cell culture being treated (Fig. 1). FE-DBD operates under the conditions where one of the electrodes is a dielectric-protected powered electrode and the second *active* electrode is a human or animal skin or organ – without human or animal skin or tissue surface present discharge does not ignite [16]. In the FE-DBD setup, the second electrode (a human, for example) is not grounded and remains at a floating potential. The principle of operation of the FE-DBD has been recently discussed in detail by the authors [16]. Of note is the fact that FE-DBD is completely safe from the electrical perspective and non-damaging for application to animal or human skin [16, 58]. Discharge ignites when the powered electrode approaches the surface to be treated at a distance (discharge gap) less than about 3 mm, depending on the form, duration, and polarity of the driving voltage.

Power deposited into plasma discharge gap was analyzed by measuring current passing through the discharge gap and the voltage drop in the gap. For current measurements, a magnetic core Pearson current probe was utilized (1 V/A +1/–0%

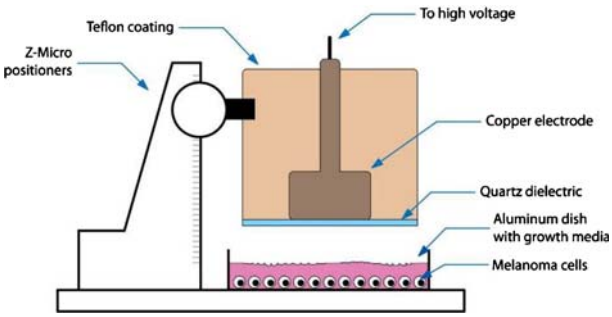

Fig. 1 Setup schematic for treatment of Melanoma cancer cells

sensitivity, 10 ns usable rise time, 35 MHz bandwidth). Voltage was measured using a wide bandwidth voltage probe (PVM-4 130 MHz 1000:1 <5% error, North Star High Voltage, Marana, AZ). Signals from the current and voltage probes were acquired and recorded by a Digital Phosphor Oscilloscope (DPS) (500 MHz bandwidth, 5×10^9 samples/sec, TDS5052B, Tektronix, Inc.). Acquired data was then processed using customized MATLAB code. In all experiments presented in this paper the plasma power was kept at 4 ±1 W, corresponding to power density of 0.8 ± 0.2 W/cm^2.

Preparation and Treatment of Melanoma Skin Cancer Cells

Melanoma cancer cell line (ATCC A2058) was propagated in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 90%, and fetal bovine serum, 10%. Cells were incubated at 37 °C with 95% humidified air and 5% carbon dioxide (CO_2). The media was changed every 2–3 days. After 4–5 days of incubation the cells were detached from the dish surface by incubating in 0.25% weight/volume (w/v) Trypsin in 0.53 mM ethylene diamine tetraacetic acid (EDTA). After detachment, trypsin was neutralized by FCS-containing growth medium. Cells were harvested by centrifugation and transferred to aluminum weighing dishes in dilution ratios of 1:5–1:6 where they were incubated as described above for 4–5 more days. The number of cells in each dish at the end of incubation was about 1.5×10^6 on average. Viability of cultures was determined by Trypan blue exclusion (see below) to be 91–97% (Fig. 2). Control and treated cells are cultured in aluminum dishes and cell health is tightly monitored. The aluminum dishes need to be thoroughly sterilized prior to the cell culturing procedures. These dishes are sterilized by alcohol and then put in the high temperature autoclave for 3 h at 350 °C where aluminum builds up a layer of oxide which prevents cells from contacting metal—this allows for higher number of cells to grow. Prior to plasma treatment, media from each dish was removed completely and a small amount (200 μL) of fresh media was added to keep cells wet during treatment. All experiments reported on in this paper were performed with 200 μL of media; separately, we have verified effect of plasma on these cells through a thicker layer of media (see Sect. 5 below and Fig. 7). Immediately following this procedure, the cells were treated with plasma for 5, 10, 15, 20, or 30 s. The distance from the electrode surface to the fluid surface was 3 ± 0.5 mm. After the treatment media was removed from the dishes, the culture was allowed to propagate further by adding

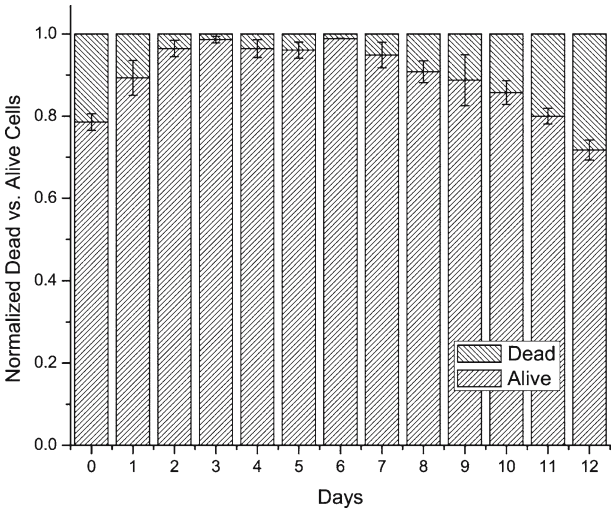

Fig. 2 Growth pattern of Melanoma cancer cells in aluminum dishes

2 mL of the fresh media or harvested by trypsinization for further testing. Trypsinized cells were scraped and collected in 15 mL tubes, centrifuged at 1,000 rpm for 10 min at 4 °C. Supernatants were removed and the cell pellet resuspended in 5 mL of 1 × Phosphate Buffered Saline (PBS).

For trypan blue exclusion, aliquots of the cell suspension were mixed with an equal volume of trypan blue (0.4% w/v in PBS, Cambrex Corp, Baltimore, MD) and transferred to a Hemocytometer slide. Live cells with intact cell membrane exclude the dye and are unstained. Total number of cells and percent of viable cells were determined by counting stained and unstained cells. Trypan Blue exclusion test was performed at different time periods after treatment: immediately following treatment, 1, 3, 24, 48, and 72 h following treatment.

Apoptosis Assay

Another group of experiments was performed, testing cells for the onset of apoptosis. Cell propagation and plasma treatment were performed as above, except that the dose of plasma for this experiment was 5 s, as this dose was found to inactivate the least number of cells. Following treatment, cells were harvested as above at 24, 48 and 72 h after treatment and DeadEnd™ Fluorometric TUNEL System apoptosis test was performed (Promega, Madison, WI) [59, 60]. This test detects apoptotic DNA fragmentation by catalytically incorporating fluorescein-12-dUTP[(a)] at 3′-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT), which forms a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. The fluorescein-12-dUTP-labeled DNA can then be visualized directly by fluorescence microscopy or quantified by flow cytometry [59, 60]. For fluorescence microscopy, cells were fixed on Lab-Tek slides for the TUNEL assay. Samples were analyzed in a fluorescence microscope using a standard fluorescence filter set to view the green fluorescence of Fluorescein at 520 ± 20 nm. Both control and treated cells were cultured in aluminum dishes to analyze the possibility of apoptosis developing from contact with aluminum.

Minimum levels of cell death were detected in control dishes that were not subjected to plasma (see Fig. 2 for example).

For flow cytometry analysis, cell suspensions were treated with the DeadEnd™ Fluorometric TUNEL System (Promega US Co), following the manufacturer's instructions. Counterstaining was done by incubating cells for 20 min at room temperature in the dark in phosphate-buffered saline containing 6 μg/mL RNAse (Roche Applies Sciences, Indianapolis, IN) and 5 μg/mL propidium iodide (Invitrogen, Carlsbad, CA) in PBS. Suspensions were washed once in PBS and resuspended in PBS for analysis. Flow cytometry was performed using a FACSORT flow cytometer (BD Biosciences, San Diego, CA) with 488 nm excitation from an argon ion laser at 15 mW. Forward scatter threshold was set to exclude small debris. Fluorescein fluorescence was captured on the FL1 channel (Fig. 11) equipped with a 530 nm wavelength filter with 30 nm bandwidth in log mode, and propidium iodide fluorescence with a 585 nm filter and 42 nm bandwidth in linear mode. Data acquisition was done using Lysis II software (version 2.0, BD Biosciences). Fluorescence spill-over was removed by compensation. Photomultiplier sensitivity was adjusted so that control cell FL1 fluorescence appeared in the first log, and PI fluorescence at approximately 200 counts (Fig. 11). At least ten thousand events were acquired per sample. Data analysis was performed using WinMDI software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, available online from http://www.facs.scripps.edu). Apoptotic and necrotic cells were differentiated by plotting PI fluorescence over fluorescein in a bivariate plot. Quadrants were set to define TUNEL-negative cells with normal DNA content; TUNEL-positive cells were counted as apoptotic, TUNEL-negative cells with lower DNA content as necrotic.

Inactivation of Melanoma Cells by FE-DBD

Melanoma cancer cell lines were treated on day 3 after initial seeding. Both control and treated cells were cultured in aluminum dishes. Immediately after seeding, there is an increase in the percentage of inactive cells but they return to their normal life cycle on day 3. Proliferation of the cells continue for 5 more days at which point their numbers become overwhelmingly high and the cells start to die (Fig. 2). Melanoma cell growth patterns are important to note, because the inactivation results reported here are not due to lack of nutrition, cell age, or the influence of aluminum substrate on the cell's life cycle. Figure 3 demonstrates cell survival numbers after 5, 10, 20, and 30 s of treatment compared to control analyzed by Trypan blue exclusion test (Fig. 4). Total cell numbers are normalized to 1 (100%) to account for cell growth between the counting sessions: controls are set to 100% and cell viability is expressed as percent to control to allow for comparison between experiments. It is of no great surprise that FE-DBD plasma is able to kill cells; what is unusual is that 24 h following treatment the total number of cells continues to decrease significantly (Fig. 3). This is potentially indicative of some internal biochemical processes taking place that FE-DBD plasma treatment had initiated, for example apoptosis or similar cell behavior.

It is important to distinguish between cell death by ''poisoning'' of the growth media the cells are in and the actual effect of direct plasma on these cells. The ability of plasma to increase acidity of the treated liquid is well known (see [25, 61], for example), and changing pH of the growth media is known to disrupt cell functions

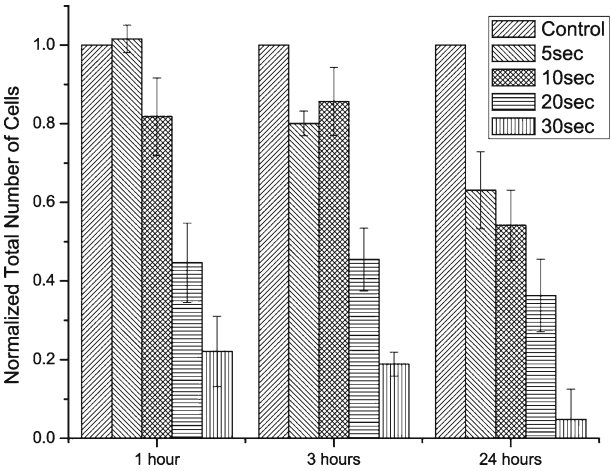

Fig. 3 Results of FE-DBD treatment of Melanoma cancer cells: Control, 5, 10, 20, and 30 s, counted 1, 3, and 24 h post-treatment

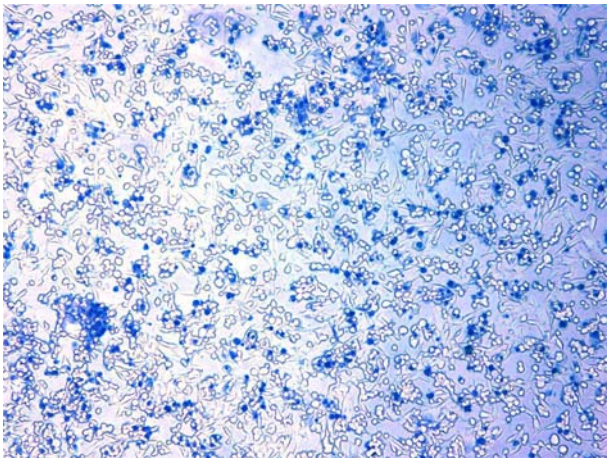

Fig. 4 Trypan blue exclusion test—blue cells (dark spots) have taken up the dye, indicating compromised cell membrane and are considered dead. Treatment time: 5 s

like metabolism, growth, etc [2, 60]. To assess the difference, growth media was treated by plasma separately to change its pH and then the cells were placed in this acidified media. Thirty seconds of FE-DBD treatment of media (same volume as is used for cell growth) changes its pH from the biologically suitable 8.5 to deadly-to-cells 5.5 (Fig. 5). As can be seen from Fig. 6, cells placed in this acidified media survived the process and the cells treated by plasma in the same volume of liquid were inactivated rapidly. Thus, we can assume that major effect of plasma on Melanoma cells is not through ''poisoning'' the growth media. Moreover, Melanoma cancer cells can withstand pH drop to 5.5 for a period that is quite longer than what is considered deadly for other mammalian cells (see [1, 60, 62], for example). Additionally, cell inactivation under varying depths of growth media was investigated. Varying depths of liquid can potentially protect the cells that are attached to the bottom of the dish during the short treatment times employed. Figure 7 clearly shows that even though, as expected, liquid protects the cells from plasma, the treatment is able to penetrate through nearly a half of a millimeter of the growth media.

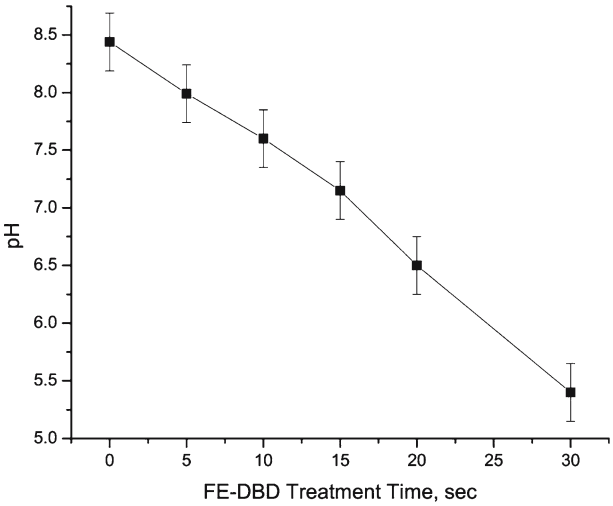

Fig. 5 Change of pH of the cell growth media treated with FE-DBD plasma 0–30 s

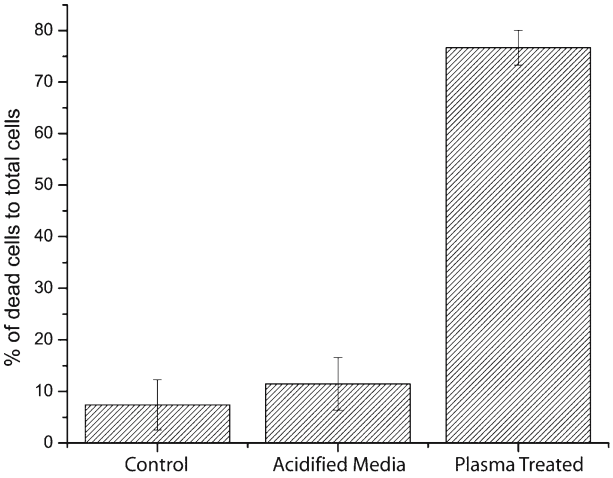

Fig. 6 Percent of dead cells in control, cell placed in acidified media for 1 min (pH 5.4), and cells treated by FE-DBD plasma for 10 s

Melanoma Cell Apoptotic Behavior

Melanoma cells, treated by plasma, immediately show evidence of cell necrosis—an expected outcome in the tradition of the recent trend of surface sterilization by plasma (see [35, 63–68] for example). However, the remaining cells that survived the treatment do not behave as the untreated cells do. The general trend observed in treated cells is that they continue to die for days after the treatment. Figure 8 presents an observation of groups of cells treated by plasma for 5 s and observed for a 3-day period following treatment. An emergent pattern appears where growth rate of treated cells is impaired as well as the number of inactivated cells grows substantially. Figure 9 shows the percentage of inactivated (dead) cells among treated and untreated populations. Thus, it was observed that 5 s of plasma treatment does not inactivate cells immediately; however, cell growth slows down significantly, and

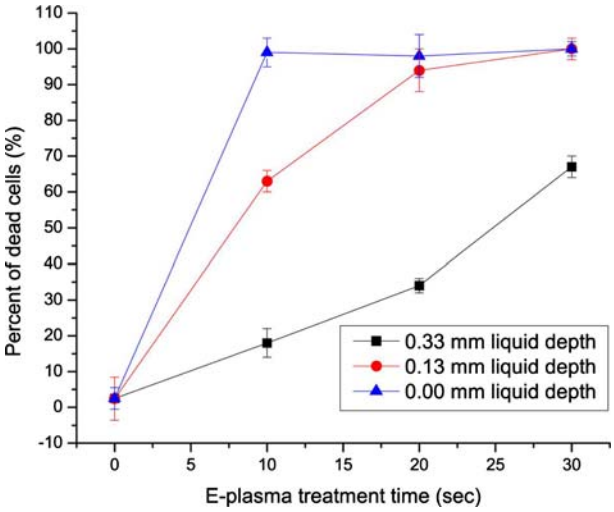

Fig. 7 Results of the FE-DBD treatment of Melanoma cells protected by varying depths of cell growth media (liquid)

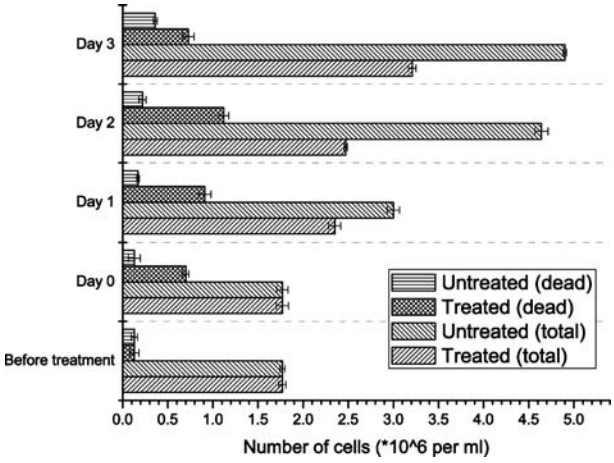

Fig. 8 Results of observation of treated and untreated cells for a three-day period: total number of cells before and after FE-DBD treatment. Treatment time: 5 s

the number of dead cells increases 24 h after treatment, which is indicative of cell death occurring long after the treatment.

To analyze whether those plasma-treated cells that survive the initial insult die through an apoptosis-like process, TUNEL assays were performed. Cells treated for 5 s were then incubated and stained for DNA fractionation 24 h later. Following the TUNEL assay procedure (see Sect. 4 above) it was observed that a significant percentage of these cells exhibit apoptotic behavior as is evident from Figure 10. The results of the flow cytometry tests performed 24, 48, and 72 h following treatment are presented in Fig. 11. Apoptosis develops 24 hours following treatment, where 25.5% of cells are present in the treated group, compared with 2.2% in the control group. As time progresses, even more cells undergo apoptosis, further reaching 72.8% of apoptotic cells in the treatment group vs. 3.2% in the control group 72 h following treatment.

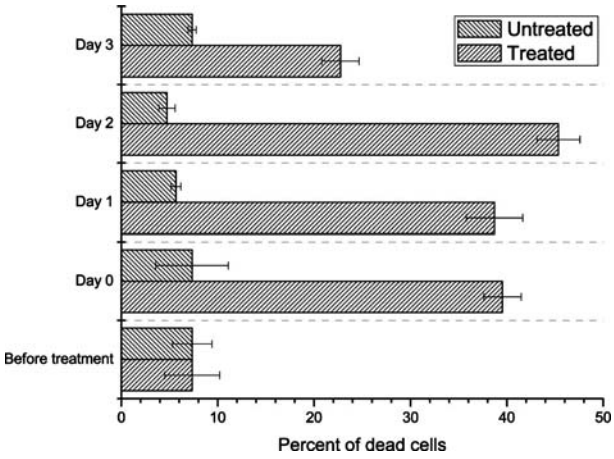

Fig. 9 Results of observation of treated and untreated cells for a 3-day period: percent of dead cells before and after FE-DBD treatment. Treatment time: 5 s

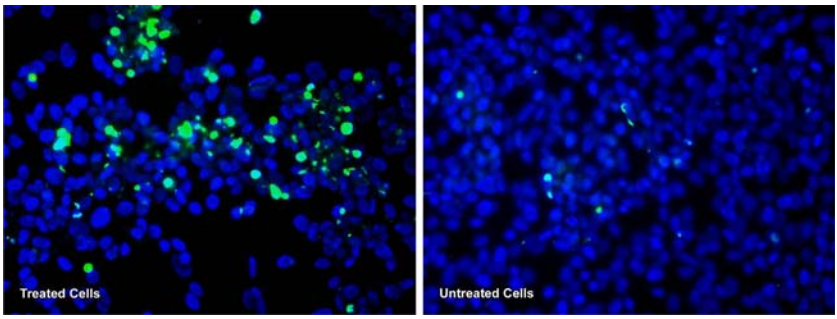

Fig. 10 Images of treated and untreated Melanoma cancer cells stained following TUNEL assay protocol. All cells are stained blue (darker circles) and apoptotic cells are also stained green (bright spots). Treatment time: 5 s; assay performed 24 h following treatment

Conclusion

FE-DBD plasma was shown to kill Melanoma skin cancer cells through *necrosis* at higher treatment doses (15 s and over at 1.4 W/cm^2 of plasma treatment) which are still below the threshold of damaging healthy tissue [16]. Very low doses of FE-DBD (5 s at 0.8 W/cm^2 of plasma treatment) where no cell necrosis was observed were shown to initiate *apoptotic behavior*, or programmed cell death in Melanoma cancer cells. During apoptosis, cells undergo a series of complex biochemical changes leading to cell death without causing inflammation. Apoptotic behavior was deduced from the fact that treated cells do not initially die but stop growth and die en masse 12–24 h following treatment, while untreated cells continue to grow and proliferate. Apoptotic behavior was confirmed through DeadEnd^{TM} Fluorometric TUNEL System apoptosis staining with subsequent flow cytometry. It was shown that the plasma treatment initiates this behavior in cells not through poisoning of the growth media in which the cells reside or through interaction with the aluminum dishes the cells reside in, but through direct interaction with the cells.

Previously it was shown by authors [16] and by other groups (see [35, 63–71]) that plasma is able to *destroy* cells; however, it was also observed that plasma might be

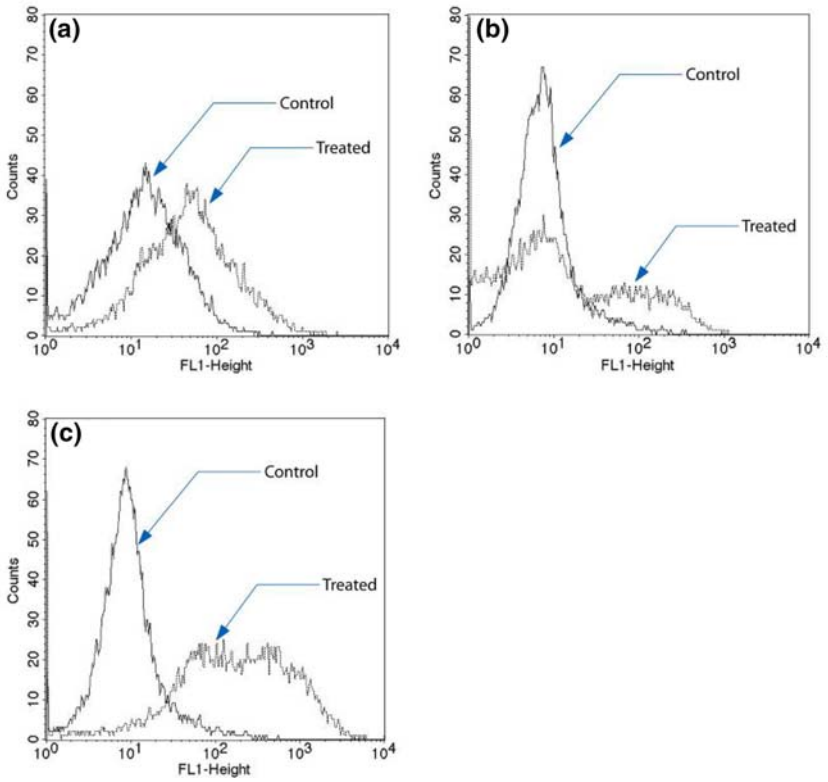

Fig. 11 Results of flow cytometry of the treated and untreated Melanoma cancer cells stained following TUNEL assay protocol. Treatment time: 5 s; assay performed 24 (**a**), 48 (**b**), and 72 (**c**) h following treatment. The FL1 (fluorescein) fluorescence histogram (number of events over fluorescence intensity) of a representative experiment is shown. Increased fluorescence intensity in treated cells is indicative of apoptotic DNA cleavage

able to initiate or *catalyze* some biochemical processes in biological systems. This is an initial step toward understanding mechanisms by which non-thermal atmospheric pressure discharge *in direct contact with cells* is able to influence their activity. Previous and presented results may be promising, but quite a few unanswered questions remain. Deeper understanding of plasma-cell interaction and of the specific biochemistry is needed to answer how plasma promotes apoptotic behavior. It is important to separate all of the effects and constituents of plasma in direct contact with cells and to analyze these constituents individually as well as to study synergetic effects of and between different plasma components to potentially receive further insight into the plasma-chemical interaction mechanisms. Understanding of the apoptotic biochemical pathways invoked by plasma, which species generated by plasma are able to invoke these pathways, and how these mechanisms are invoked is also essential. Future work will primarily address fundamental understanding of plasma interaction with living tissue and physical and biochemical mechanisms thereof, for example effect of various plasma-generated excited species on the cell membrane and membrane proteins.

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