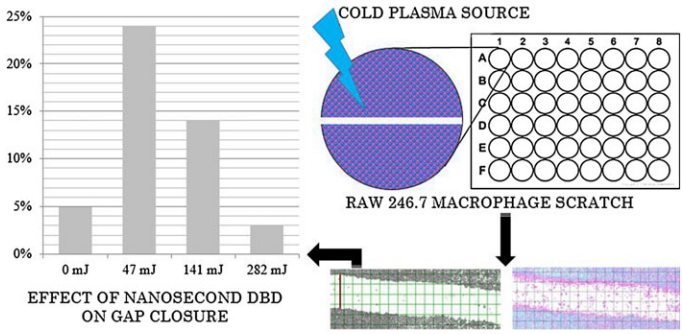
Plasma Stimulation of Migration of Macrophages

Vandana Miller,* Abraham Lin, Gregory Fridman, Danil Dobrynin, Alexander Fridman

Plasma has been shown to be effective against cancer cells both in vitro and in vivo. Several studies have documented the selectivity of plasma against cancer cells with minimal or reduced damage to normal cells. However, complete remission of cancer following plasma treatment has not been achieved yet. In the body, the immune system plays a vital role in the prevention and control of cancer. Presence of cells of the adaptive immune system in the tumor microenvironment is usually an indicator of good prognosis.[1] Since immune cell migration is a key initial step towards defense against diseases, it is important to evaluate the influence of plasma treatment on such cellular functions. Here we show that treatment with nanosecond-pulsed non-thermal dielectric barrier discharge enhances migration of macrophages in vitro.



1. Introduction

It is now an accepted fact that non-thermal plasma kills cancer cells in vitro.[2–5] Recent evidence also demonstrates that tumor masses decrease in size in vivo following plasma treatment and that life expectancy of treated animals is increased.[4,6,7] However, complete remission has not yet been achieved. One reason for this may be the non-penetrating nature of plasma that makes direct delivery to affected tissues a key challenge. Nevertheless, a reduction in tumor size clearly indicates that plasma produces some effect.

Cancerous masses are more than just a mass of mutated cells. They contain epithelial cells, fibroblasts, blood vessels, lymphatics and immune cells. They also harbor mesenchymal cells that create a microenvironment that is unique to each cancer. These cells communicate with each other to maintain homeostasis just like any other tissue; the only difference is that this tissue is under the control of mutated cells that promote the survival of this mass at the cost of other normal tissues.[8]

Cancer results from uncontrolled cell division due to mutations; no single gene defect results in cancer. The failure of the immune system to eliminate these mutated cells is the additional important step that allows the transformed cells to grow unchecked.[9] In the early 20th century Paul Ehrlich first suggested that cancer would be more prevalent were it not for the protective effect of the immune system.[10] We now know that tumor cells are recognized as "foreign" or "non-self" by a process called immunosurveillance and eliminated by cells of the immune system.[11] Dendritic Cells, Macrophages, Neutrophils and Mast Cells reside in tissues and continuously survey the environment for such signals. These cells then recruit cells of the specific immune system — T- and B-lymphocytes — that restrain and orchestrate the destruction of the outgrowth. Failure of the immune system to respond at any of these stages favors tumor development.[1] Tumor cells may also evolve to evade recognition as "foreign" or become "invisible" to immune cells and grow into malignant tumor masses.

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In experimental models of tumors, the importance of various cells of the immune system in harnessing tumors has been clearly demonstrated. Pioneering experiments by Ruco et al in 1978, demonstrated the cytotoxic anti-tumor effects of activated macrophages.[12] In several subsequent studies this macrophage mediated tumor regression was attributed to secreted factors like Tumor Necrosis Factor (TNF) and Interleukin 1 (IL-1) as well as to direct cell-cell contact.[13] The protective role of macrophages in cancer is well summarized in a 1993 review by Banta and Ben-Efraim.[14]

It is evidently clear that macrophages serve as key cells in anti-tumor reactions in the body, functioning at every stage of immunological responses. Beginning with recognition of tumor cells as foreign, to processing and presentation of tumor antigens to T- and B-lymphocytes, production of soluble mediators that are toxic to cancer cells and finally, killing of tumor cells through direct contact, these cells are indispensable for effective tumor elimination. We therefore questioned if it is possible to influence the functional capabilities of macrophages by exposure to nanosecond-pulsed dielectric barrier discharge plasma. In this paper we demonstrate that plasma treatment is successful in activation of macrophage function in vitro. While most cells in the body remain in place, immune cells are able to travel from the blood vessels into tissues when required. In fact, this migratory capability is vital for them to be effective for host defense against any foreign assaults.[15] This is the first report where non-thermal plasma treatment has been shown to influence a macrophage function, namely migration.

2. Experimental Section

2.1. Cell Culture

The RAW 264.7 mouse macrophage cell line was a kind gift from Dr. Michael Autieri (Temple University, Philadelphia, PA). Cells were maintained in Dulbeccos's Modified Eagles Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin by volume in tissue culture treated 75 cm^2 flasks. Flasks were incubated at 37 °C and 5% relative humidity. To make subcultures, cells were dislodged from culture flasks with a cell scraper and reseeded at 1:3 to1:6 dilution twice a week.

For the viability and scratch assays cells were scraped and transferred into a centrifuge tube. They were centrifuged at 200 × g for 10 min. Following a cell count using the trypan blue exclusion method, they were resuspended in fresh media at a final concentration of 5×10^5/ml. One ml of this suspension was plated in each well of a 24-well plate and allowed to grow overnight to allow adherence to the plate bottom.

2.2. Plasma Treatment

A nanosecond-pulsed dielectric barrier discharge (nsDBD) plasma source was used for all cell treatments (Figure 1). nsDBD plasma

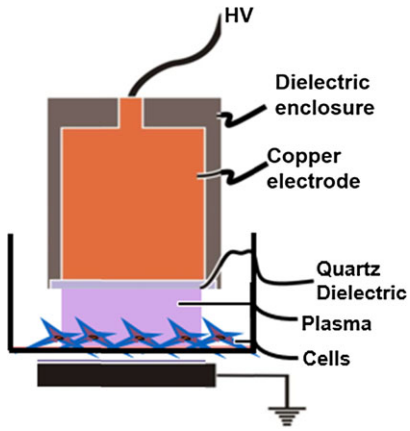


Figure 1. Schematic of Plasma delivery to cells.

was produced by applying a positive pulsed voltage of 29.4 kV between the high voltage electrode and the cells. The electrode was positioned at 1 mm distance from the cells using a Z-positioner and the treatment duration was set at 10 seconds for all experiments. All plasma treatments were done in the absence of any media. The frequencies used were 5, 15, 30 and 75 Hz corresponding to delivered energies of 47, 141, 282 and 705 mJ respectively.

2.3. Cell Viability Assay

The viability of cells was evaluated one-hour post treatment. Cells were stained with 100 μg/mL Propidium Iodide (Invitrogen, NY), a DNA binding fluorescent reagent that only permeates damaged cell membranes. Cell viability and concentration was determined using image cytometry (Nexcelom Bioscience, USA) and further analysis was performed with the FCS flow cytometry software.

2.4. Scratch Assay

To begin, each well of the 24-well plate was carefully examined to ensure that a confluent monolayer of cells was established. A clean scratch was made in the confluent monolayer of the RAW cells, using a p200 tip, as described.[16] Wells were washed twice with serum-free PBS containing Calcium and Magnesium to remove floating cells. PBS was removed and cells were treated with nsDBD at frequencies listed above, immediately following the wash steps. The cells were then fed with DMEM containing 2% FBS to inhibit cell proliferation. The experiment was set up in triplicates. Each scratch was visualized using a Nikon Eclipse TE-2000-S microscope and images were captured with a S.P.O.T. camera. The plate was incubated for 24 h and images were captured again. Each image was analyzed using S.P.O.T. software and scored for gap distance and number of cells in the gap area at time 0 (T0) and 24 hours (T24) after scratch and treatment. For gap closure, the gap was measured on images from the same area of each well and the difference represented gap closure. Since the scratch was not identical from well to well, this was converted into percent closure comparing to gap distance at T0.

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3. Results and Discussion

3.1. Plasma Dose and Cell Viability

The goal of this study was to establish a non-lethal plasma treatment regimen for the RAW 264.7 cells so that any changes from the untreated controls could be solely attributed to cellular migration and not change in cell numbers. Each well was treated with nsDBD plasma at frequencies of 5, 15, 30 and 75 Hz for 10 s. The voltage was set at 29.4 kV for each treatment and the electrode was positioned at 1 mm from the cells. This treatment ensured delivery of uniform plasma. One hour after treatment, cells were stained with PI to determine the number of viable cells. These were then compared to the number of viable cells in the non-plasma treated control wells and are represented as percent change from controls (Figure 2 and Table 1).

Table 1. Recovery of RAW 264.7 cells 24 hours post treatment RAW 264.7 macrophages were treated with nsDBD (29.4 kV) for 10 seconds at a distance of 1 mm at the indicated frequencies, in the absence of any tissue culture fluid. Viability was measured 1 and 24 hours after treatment using Propidium Iodide and quantified with the Cellometer. The live cells had no residual effects and continued to replicate, as seen in Table 1. Experiments were performed in triplicates.

Frequency	Fold increase in cell number after 24-hour incubation
0 mJ	2.26 ± 0.14
47 mJ	2.94 ± 0.63
141 mJ	2.42 ± 0.27
282 mJ	2.83 ± 0.34

3.2. Scratch Test Analysis

In this experiment, we wanted to examine the effect of nsDBD treatment on cellular migration in an in vitro wound-healing model, the scratch assay. When a scratch is made in a monolayer, the cells at the edge of the "wound" try to close the gap until new cell-to-cell contact is established. This assay is a cheap and reasonable simulation of cell migration in vivo. An artificial gap was created across the RAW cell monolayer using a p200 pipette tip immediately prior to plasma treatment. Images were captured for each well immediately after plasma treatment. The plates were then allowed to incubate at 37 °C in DMEM containing 2% FBS.[16] The reduction in serum concentration ensures that no cell proliferation occurs and the gap closure is attributed to migrating cells only. A second set of images was captured after 24 h of incubation. Representative images are shown in Figure 3.

Each image was analyzed using S.P.O.T. software and scored for gap distance and number of cells in the gap area at time T0 and T24 after scratch and treatment. For gap closure, the gap was measured on images from the same area of each well at twenty different points and averaged. The difference between the T0 measurements and the 24-hour measurements represented gap closure. Since the scratch was not identical from well to well, this was converted into percent closure comparing gap distance at T0. As seen in Figure 4 there is some narrowing of the gap even in the absence of plasma treatment. However, exposure to nsDBD at 5 Hz and 15 Hz resulted in an enhanced response by the cells to reduce the gap distance. To count the number of cells in the gap, color-coded T0 images of each scratch were overlaid on to the T24 images. The number of cells in the T0 gap area was counted as a reflection of migrating cells. The results are represented in Figure 5 as percent change compared to the untreated controls. Both 5 and 15 Hz treatments resulted in an enhanced migration

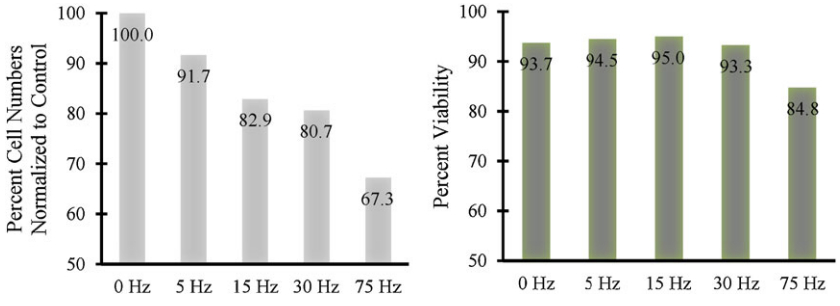


Figure 2. Effect of nanosecond pulsed DBD on viability of RAW 264.7 cells. RAW 264.7 macrophages were treated with nsDBD (29.4 kV) for 10 seconds at a distance of 1 mm at the indicated frequencies, in the absence of any tissue culture fluid. Viability was measured 1 hour after treatment using Propidium Iodide and quantified with the Cellometer. The total numbers of intact cells (Left) decreased with increasing frequency. However the viability of the intact cells remained unchanged at lower frequencies (Right). The live cells had no residual effects and continued to replicate, as seen in Table 1. Experiments were performed in triplicates.

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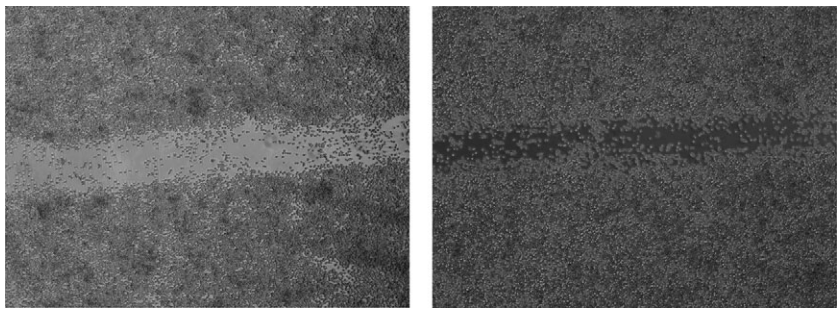


Figure 3. Representative images of scratch after 24 hours. The scratch treated with 47mJ nsDBD plasma (right) has greater gap closure and more migrating cells in the gap as compared to the untreated scratch (left).

of macrophages. At 30 Hz treatment there was a marginal decrease in gap closure effect and a significant decrease in the number of migrating cells. We hypothesize that this may be due to increased cell loss (lysis and detachment) under this treatment condition.

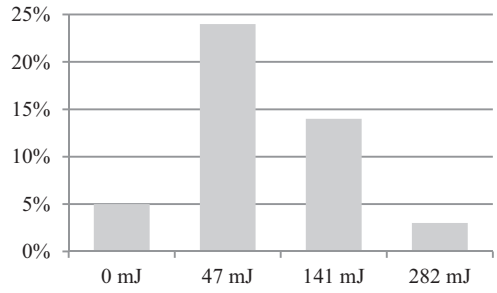


Figure 4. Effect of nanosecond DBD on gap closure Gap closure was assessed for each condition by comparing the gap distance at time 0 and at 24 hours post scratch and treatment. A 10 sec treatment at 5 Hz resulted in maximum gap closure.

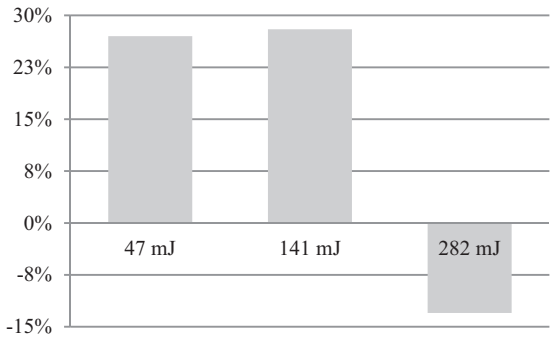


Figure 5. Effect of nanosecond DBD on the number of migrating cells in the gap. The number of migrated cells in the gap are represented as a percent of untreated controls. 5 Hz and 15 Hz treatments resulted in a 27% and 28% increase.

4. Conclusion

We demonstrate the ability of nsDBD plasma to stimulate macrophages in vitro to attempt healing of an artificial wound. Treatment of scratched wells with nsDBD at 5 Hz and 15 Hz produced approximately 25% increase in migrating cells. Gap closure was also enhanced at these treatment frequencies. This suggests that it is possible to stimulate macrophage function in vivo using appropriate plasma regimes. Stimulated macrophages could then be available for tackling infectious organisms, cancers, or any other assault. In fact, re-examination of studies by Keidar et al [4,7], in this light, suggests the involvement of immune cells. Nude mice, used in their study [4] have a genetic defect that results in a lack of T-lymphocytes, a key cell type in the adaptive tumor response. When injected with B6 melanoma cells, these mice survived about 24.5 days. Plasma treatment extended their life-span by 9 days to 33.5 days.[4] The same experiments in normal mice, with an intact immune system, resulted in increased median life expectancy of 20 days.[17] Since the animals were on the same genetic background and the only difference was the presence of T-lymphocytes, it is reasonable to conclude that plasma treatment boosted immune function.

Our studies provide preliminary evidence that non-thermal plasma is efficacious in stimulation of certain functions of macrophages. Further studies are required to examine other macrophage functions both in vitro and in vivo, such as phagocytosis, bacterial killing, production of cytokines etc.

Our studies suggest that non-thermal plasma mediated stimulation of immune function may be a viable alternative for cancer therapy and must be explored further.

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