Analyzing the Possible Action Mechanisms of Atmospheric Pressure Nonthermal Plasma upon Melanoma Cells

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ABSTRACT: Cancer refers to a group of diseases characterized by an uncontrolled growth of aberrant cells as a consequence of multiple genetic alterations; melanoma is considered the most dangerous of all types of skin cancer due to its capability to produce metastasis and chemoresistance. Recently, several studies point to atmospheric pressure nonthermal plasma (APNTP) as a good alternative for cancer treatment. The effect of APNTP upon cells has been largely attributed to the generation of reactive oxygen and nitrogen species that act upon biomolecules; however, there are some other factors that could contribute to the full effect on cells or tissues. The main objective of the present work is to contribute to the understanding of the action mechanisms of nonthermal plasma upon cancer cells. For this work, B16 murine melanoma and 3T3 murine fibroblasts cells were used, as well as human lymphocytes. B16 and 3T3 cells were grown in MEM with 10% FBS at 37°C and 5% CO2 atmosphere, while human lymphocytes were separated from total blood by using the Ficoll Hypaque technique. Cells were resuspended in HBSS, distributed in a 96-microwell plate, and exposed to APNTP. To evaluate the possible membrane disruption, cells were counted before and immediately after plasma exposure considering that, if the membrane was crashed, they could not be seen under the microscope. The presence of intracellular oxygen species was determined by fluorescence microscopy using the dihydrorhodamine/ethidium bromide test. The effect of UV-B component of plasma upon DNA was evaluated by a modified microelectrophoresis (Comet) assay. The results indicate that the number of cells considerably decreased due to membrane disruption; also, the presence of ROS was confirmed in the cytoplasm of treated cells, as well as DNA damage generated by the UV-B fraction of APNTP, which in turn could be a factor triggering apoptosis.

KEY WORDS: APNTP, action mechanisms, melanoma, RONS, UV-B light fraction

I. INTRODUCTION

The term cancer refers to a group of diseases characterized by the uncontrolled growth of aberrant cells as a consequence of multiple genetic alterations upon genes related to cell cycle regulation. To date, cancer is one of the most critical health issues worldwide, causing about 9.5 million deaths in 2018. Melanoma is considered the most dangerous of all types of skin cancer due to its capability to metastasize and to develop
Acording to GLOBOCAN, in 2018 over 285,000 cases of melanoma and about 60,000 deceases were reported around the world, so it is important to keep searching for new treatments. Due to its location, Mexico receives relatively high amounts of UV radiation, thus increasing the risk of exposure of its population to this agent, which is prone to cause genetic alterations, mainly cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP) that can generate mutations, which in turn could give rise to cancer. Usually, cancer is treated by surgery or immuno-, chemo-, or radiotherapy, but unfortunately these treatments have many side effects, making this process more difficult to carry out. Recently, atmospheric pressure nonthermal plasma (APNTP) has arisen as a new and effective option to fight this disease.

Plasma is a partially ionized gas containing mostly stable molecules and a fraction of free ions and electrons, radicals, and excited molecules. Plasma can be categorized based on the thermal equilibrium between electrons and ions. In thermal plasma, the temperature between the heavy ions and the electrons is the same; that is, they are in thermodynamic equilibrium. When the electron’s temperature is much higher than that of the heavy species, it corresponds to nonthermal plasma. This type of plasma can be generated at atmospheric pressure in different ways. One of them is dielectric barrier discharge (DBD) produced by the electric discharge between two electrodes separated by a small distance. One of its electrodes is covered with a dielectric to prevent the plasma discharge from turning into an electric arc. For the generation of nonthermal plasma, a noble gas is used. The electrodes are polarized with power sources such as pulsed, alternating current, radio frequency (RF), or microwaves. Usually, in a coaxial configuration, one of the external electrodes is outside the chamber, and the other electrode is coated with a dielectric placed inside the chamber, and the noble gas is injected. The electrodes are polarized by one of the polarization sources indicated above. An intense electric field is generated that causes the dielectric breakdown of the molecules’ gas, producing the atmospheric pressure nonthermal plasma (APNTP). The low working temperature of this type of plasma allows the treatment of heat-sensitive material and its use on living tissues.

It can be generated by applying a radiofrequency electric power to a confined flow of a gas, like argon, helium, air, etc. To date, APNTP has been tested for many different applications such as disinfection, wound healing, blood coagulation, and tissue regeneration. Moreover, APNTP has been demonstrated to be safe when it is used in promoting wound healing and even healing ulcers in diabetic patients, and in most cases it reduces the pain sensation of patients while reducing inflammatory processes.

Recently, several studies point to nonthermal atmospheric plasma as a good alternative for cancer treatment. The effect of APNTP upon cells has been largely attributed to the generation of reactive oxygen and nitrogen species (RONS) that can act against several biomolecules such as membrane phospholipids or nucleic acids. However there are some other factors that could contribute to the full effect of APNTP on cells or tissues. The main objective of the present work is to contribute to the understanding of the action mechanisms of nonthermal plasma upon cancer cells.
II. METHODOLOGY

A. Cell Culture

For this work, B16 murine melanoma and 3T3 murine fibroblasts cells were used, as well as human lymphocytes. Lymphocytes were included in the systems to have a cell type that was not in constant division so by comparison, the role of DNA replication in the effect of APNTP could be inferred; however, many mice would had to be used to have the volume of blood needed for the experiments, so we decided to use human blood instead. Cells were grown in either Dulbecco’s modified minimal essential medium for B16 or minimal essential medium for 3T3, with 10% FBS at 37°C and 5% CO₂ atmosphere. Cells were harvested by tripsinization, washed twice with Hanks balanced saline solution, and further incubated for at least one hour in MEM at 37°C to recover from harvesting stress. Blood samples were obtained from healthy donors by venopunction, and lymphocytes were separated by using the Ficoll Hypaque technique. Nucleated cells were collected, washed twice with HBSS, and kept in RPMI-1640 with 10% FBS at 37°C for at least one hour.

B. Plasma Exposures

Cell were resuspended in HBSS and then 200 ml aliquots were distributed in a 96-microwell plate and exposed to APNTP generated by a spot application reactor supplied with a flow of 1 LPM of helium that is excited through a 13.56-MHz radiofrequency generator with a set load power up of 10 W and coupled via a homemade “Pi” matching network (Fig. 1). Table 1 correlates the energy delivered and the different times used.

C. Membrane Disruption

To evaluate the possible membrane disruption, cells were counted before and immediately after plasma exposure considering that, if the membrane was totally crashed, cells could not be seen under the microscope.

D. Intracellular ROS

The presence of intracellular oxygen species was determined by fluorescence microscopy using the dihydrorhodamine/ethidium bromide test; after exposure, cells were resuspended in 50 ml of HBSS and then dihydrorhodamine 123 (DHR₁₂₃) was added at a final concentration of 1 mM and further incubated for 20–30 min. Cell suspension was washed-again and resuspended in the same volume of 30% glycerol in MEM with 30 μM propidium iodide. The cells were observed with an epifluorescence microscope using a 488-nm excitation filter. A semiquantitative score was made, sorting the cells
into three different categories according to the color displayed: normal cells exhibited a faint green color, oxidized cells showed a bright green color, and dead cells were stained in red.

**TABLE 1:** Energy delivered at the different exposition times used

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>Energy (J/cm²)</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>15</td>
<td>4.63</td>
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<td>9.26</td>
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<tr>
<td>45</td>
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<td>60</td>
<td>18.52</td>
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<tr>
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<td>23.15</td>
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</tbody>
</table>

FIG. 1: Schematic diagram of the plasma exposure
E. CPD Detection

Since APNTP has a component of UV-B during the exposures, we decided to test the effect of this component upon DNA by means of a modified comet assay. After treatments, 200 μl of cell suspension were mixed with an equal volume of 1% low melting point agarose, poured on top of fully frosted microscope slides and immersed in cold lysis solution (4°C) for an hour. Then, slides were rinsed with the Tris-HCl buffer (pH 7.5), and then 40 μl of a 5 U/ml solution of pyrimidine dimer glycosyldase enzyme were applied and incubated at 37°C into a humidity chamber for 15 minutes. UV induces mainly cyclobutane pyrimidine dimer (CPD) on DNA, and this enzyme specifically recognizes these CPDs and generates abasic sites, which in turn will be converted in DNA breakage, thus increasing DNA migration. Slides were then transferred to an electrophoresis cell, covered with cold electrophoresis solution (0.3 M NaHO, 0.1 mM Na₂EDTA) for 20 minutes to allow DNA unwinding, and then a current was applied (20 V, 300 mA, 20 minutes). Slides were stained with 60 µL of ethidium bromide (20 µg/mL) and scored under an epifluorescence microscope by means of the Comet Assay IV Analyzer (Perceptive Instruments Inc.).

III. RESULTS AND DISCUSSION

The results of membrane disruption are shown in Fig. 2A. It can be seen that there is an evident decrease in the number of cells (despite the type) according to the exposure

FIG. 2: (A) Cell population decreasing as a consequence of membrane disruption after plasma exposure; (B) survival to APNTP of the different cell types used (reprinted from Serment-Guerrero et al. with permission from the Instituto de Ciencias Aplicadas y Tecnología, copyright 2019)

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time, indicating that the plasma is actually disrupting the membrane, thus destroying the cells. However, such decrease is almost the same in all three cell types, suggesting that the APNTP is affecting the membrane equally and acts randomly upon them. In previous experiments we reported a differential survival percentage to APNTP (Fig. 2B), as the melanoma cells are more sensitive to the plasma exposure. However, these experiments were performed by counting the stained cells under the fluorescence microscope. If both membrane disruption test and survival test results are merged, we can evaluate the actual cell killing produced by this agent (Table 2). For each plasma exposure, the number of the remaining intact cells calculated in the membrane disruption experiment is taken as 100%. The drop of cell population was then determined using a rule of three considering the percentage of live cells from the fluorescein diacetate experiment. This way the actual cell population reduction was calculated. The population of B16 cells descend to less than 2%, almost 1-fold lower than that observed for lymphocytes and even more in the case of fibroblasts.

It has been proposed that the main mechanism of action of the APNTP is due to generated RONS, so to have more evidence about that, we used the dihydrorhodamine/ethidium bromide technique reported by Gossens et al. DHR is a cell-permeable fluorogenic marker specific for $\text{H}_2\text{O}_2$. Oxidation of the nonfluorescent DHR by these radical species yields the fluorescent rhodamine 123. The results demonstrate that APNTP produces intracellular oxidation (Fig. 3) that in the end could react either with nucleic acids or other biomolecules or organelles, such as mitochondria. Interestingly, the generation of intracellular hydrogen peroxide is higher in the melanoma cells, in agreement with the fact that in this type of cell the oxidative species are usually elevated, most probably due to a lower expression in antioxidant enzymes.

When the plasma is produced, a small fraction of ultraviolet light (UV-B) is also generated, which could affect cells, especially DNA. As demonstrated in previous experiments, APNTP produces DNA breakage that can be detected by means of the comet assay, and most of these breaks are surely generated by RONS. However, the standard comet assay methodology cannot detect other types of DNA lesions that do not produce breakage directly, such as CPDs. The idea of adding a step with an enzyme that recognizes exclusively CPDs is to prove that the UV-B component is actually generating DNA damage that at the end could be important in the fate of the cell or tissues. The results show an increase in tail moment when the glycosidase is used, evidencing the presence of CPDs. To prove that there is DNA damage due to the UV-B fraction, we used a modified comet assay. In this, a glycosidase that specifically recognizes CPD’s is added, leaving abasic sites (AP sites) that will be converted in DNA breaks during the alkali treatment. The results indicate that there is an increase in DNA migration (expressed as tail moment) when the PDG enzyme is used and depends on the dose applied, clearly indicating that the UV-B fraction is affecting the genetic material (Fig. 4). It is worthwhile noting that such difference is much more evident in melanoma, surely because this type of cell is deficient in the nucleotide extension repair (NER) pathway, which usually recognizes and repairs this kind of DNA damage. This could contribute to explain the higher sensitivity of the melanoma cells to APNTP. Moreover, the fact that
TABLE 2: Effect of APNTP on the actual elimination of cells

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>Fibroblasts</th>
<th>Lymphocytes</th>
<th>Melanoma</th>
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<tbody>
<tr>
<td></td>
<td>Cell population (%)</td>
<td>Survival (%)</td>
<td>Actual cell reduction (%)</td>
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</table>
**FIG. 3:** Intracellular hydrogen peroxide generated by APNTP. Striped portion, normal state cells; white portion, oxidative damage; black portion, dead cells.

**FIG. 4:** Presence of CPDs in cells exposed to APNTP. Empty bars, comets without glycosidase; filled bars, comets with PDG glycosidase.
CPDs remain unrepaired in the genome of the exposed cells could in turn be a factor to trigger apoptosis, along with the oxidative damage to mitochondria.\textsuperscript{17}

IV. CONCLUSIONS

The results obtained indicate that there are several ways in which the APNTP could affect the cell. It was observed that there is a membrane disruption that eliminates part of the cell population regardless of the type of cell. The experiments with DHR\textsubscript{123}/EB confirmed the generation of intracellular hydrogen peroxide that could act upon any biomolecule or organelle. Finally, the modified comet assay demonstrated the presence of CPDs generated by the UV-B fraction of APNTP. Since melanoma cells are in constant replication and many of them are deficient in oxidative stress protection and nucleotide excision repair mechanism (which is in charge of the elimination of CPDs), this agent promises to be an excellent option for melanoma treatment.

REFERENCES


