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Intracellular effects of atmospheric-pressure plasmas on melanoma cancer cells

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Gas discharge plasmas formed at atmospheric pressure and near room temperature have recently been shown as a promising tool for cancer treatment. The mechanism of the plasma action is attributed to generation of reactive oxygen and nitrogen species, electric fields, charges, and photons. The relative importance of different modes of action of atmospheric-pressure plasmas depends on the process parameters and specific treatment objects. Hence, an in-depth understanding of biological mechanisms that underpin plasma-induced death in cancer cells is required to optimise plasma processing conditions. Here, the intracellular factors involved in the observed anti-cancer activity in melanoma Mel007 cells are studied, focusing on the effect of the plasma treatment dose on the expression of tumour suppressor protein TP73. Over-expression of TP73 causes cell growth arrest and/or apoptosis, and hence can potentially be targeted to enhance killing efficacy and selectivity of the plasma treatment. It is shown that the plasma treatment induces dose-dependent up-regulation of TP73 gene expression, resulting in significantly elevated levels of TP73 RNA and protein in plasma-treated melanoma cells. Silencing of TP73 expression by means of RNA interference inhibited the anticancer effects of the plasma, similar to the effect of caspase inhibitor z-VAD or ROS scavenger N-acetyl cysteine. These results confirm the role of TP73 protein in dose-dependent regulation of anticancer activity of atmospheric-pressure plasmas. © 2015 AIP Publishing LLC. [http://dx.doi.org/10.1063/1.4933366]

I. INTRODUCTION

Non-equilibrium gas discharge plasmas formed at atmospheric pressure and near room temperature plasmas have been attracting interest for their demonstrated ability to inactivate pathogenic microorganisms, promote tissue healing, and selectively induce apoptosis in cancer cells.1–9 The biological activity of such plasma treatment stems from a combination of plasma-induced chemical and physical processes, including contributions from electric field, ultraviolet radiation, overpressure shock waves, and formation of various reactive chemical species such as radicals and molecular species, although their exact contributions toward overall treatment efficacy remains poorly understood.10

In general, thermal and electric effects that lead to direct denaturing of the target cells and tissues are minimised in favour of chemistry-driven responses,11 with the nature and level of highly reactive chemistry, in particular, reactive oxygen and nitrogen species (RONs) deciding the fate of cells. In cancer medicine, this chemistry-specific approach enables selective killing of cancer cells with limited side effects on normal cells.12–16 Indeed, current research in the area shows that unlike conventional anti-cancer drugs, such as eribulin or cabazitaxel,17 plasma treatment can be effective in inducing programmed cell death (apoptosis), in a broad range of cancer cell types in vitro and in vivo, with no evidence of normal cell death.15,16,18–20

The nature and relative concentration of chemical species generated in plasmas vary greatly between plasma systems and as a function of processing parameters, such as pressure, background gas composition, operating frequency and power, and whether they are produced in solution, in air or at the gas/solution interface. In solutions, plasmas induce the formation of hydroxyl radical OH•, ozone (O3), and hydrogen peroxide (H2O2), which then drive the plasma-induced changes in molecules and cells via oxidation. In atmospheric-pressure plasmas, the interactions between the plasma and the air results in partial dissociation and ionization of ambient O2, N2, and H2O, giving rise to biologically relevant levels of species capable of inducing specific intracellular responses. These include oxygen species, e.g., O2, singlet delta oxygen (O2 a1Δg), H2O2, OH•, and nitrogen species, e.g., atomic nitrogen (N), nitrogen oxides (e.g., NO, NO2, and N2O), peroxynitrite (ONOO•), nitric and nitrous acid (HNO3, HNO2), and several others. When plasma is generated at gas/solution interface, the produced reactive species diffuse into the solution, where they engage into chemical reactions with treated cells or biomolecules. While ions tend to recombine in the vicinity of the visible plasma plume, neutral species can travel significantly further. In addition to oxidation, reduction induced by H• and superoxide radicals and molecular hydrogen can contribute to the plasma-induced change.

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Many of these chemical species play critical roles in the biology of normal and cancer cells. However, their significance in inducing specific intracellular biochemical events as part of plasma-cell interactions is not well understood. The balance of the species has been shown to yield very specific biological outcomes, inducing differentiation in stem cells in NO-rich acidic solutions and killing effect in basic ROS-rich solutions.

The biological outcome strongly depends on the dose of the plasma exposure, from stimulating cell proliferation at low doses to inducing apoptosis (controlled cell death), and finally, necrosis (uncontrolled cell death) as the dose is increased. The dose is broadly defined by the energy used to generate the plasma, the length of the treatment, and the proximity of the plasma glow to the treatment zone. It is important to note that not all the energy used to generate the plasma is transferred to the species that actually interact with the surface of biological objects.

However, since there is still no clear understanding of which plasma effects are most biologically relevant, the dose does not reflect the quantity of specific chemical species or physical effects required to achieve the desired biological responses. This impedes translation of plasma antitumor treatments from laboratory to clinical applications.

Equally hindering is the lack of understanding regarding the intracellular processes that take place upon plasma exposure, which limits our ability to optimise the treatment process. Our previous work on atmospheric gas plasma treatment of melanoma and healthy melanocyte cells demonstrated selective induction of apoptosis in cancer cells and described one of the mechanisms by which apoptosis was achieved (the oxidative stress–induced TNF-ASK1-JNK/p38–caspase-3/7 apoptotic pathway).

The aim of this article is to further explore the intracellular factors involved in the observed anti-cancer activity of non-equilibrium atmospheric-pressure plasmas, and to introduce some of the key biological mechanisms involved in plasma-cancer cell interaction. This article reports on the effect of plasma treatment dose on the expression of tumour suppressor protein TP73 in melanoma Mel007 cells. TP73 is a member of the p53 family of proteins that are known to induce cell cycle arrest and apoptosis in cancer cells.

II. MATERIALS AND METHODS

A. Atmospheric-pressure plasma jet

All cells were treated with atmospheric-pressure plasma plume, which was generated using a custom-designed atmospheric-pressure plasma jet device as shown in Figure 1 and described in detail previously. Briefly, the device consists of a fused quartz tube equipped with two conducting electrodes. One electrode is a metal wire placed inside the tube along tubal-axis, finishing ~1.8 cm before the nozzle exit. The second electrode is a metal ring attached to the outer wall of the tube near the nozzle exit. The apparent distance between the two electrodes is ~1.0 cm. Helium gas is flown through the quartz tube at 2 l/min. A non-equilibrium plasma discharge is produced between the two electrodes by an applied AC high voltage. The high voltage is generated by an RF power source coupled with an RF voltage amplifier. The cells treatment is conducted at the discharge voltage and operating frequency between 1.1–1.8 kV and 230–270 kHz, respectively.

The plasma discharge produced at the end of the metal wire electrode is located in two different spatial regions. One part of the discharge is located entirely inside the tube, from the end of the metal wire electrode to the ring electrode, and part of the discharge from the ring electrode propagates along with the gas flow and extends out of the tube through the nozzle as a collimated plasma jet. Under selected treatment conditions, the length of the plasma jet exceeds 2.5 cm, with the end diameter of this collimated plasma jet at ~500 μm. This plasma jet is considered a cold plasma due to the measured low gas temperature (~35–40°C).

During the discharge process, electrical parameters of the plasma, such as discharge voltage and current, are monitored using high- and low-voltage probes, respectively. The discharge current between the metal wire and the ring electrodes is 10 mA. However, only 5%–10% of this current is likely to extend out of the discharge tube, which is used for cell treatment.

B. Cell treatment and characterisation

The human melanoma cell line Mel007 was provided by Peter Hersey, Melanoma Institute, University of Sydney and were maintained in DMEM media (Invitrogen) plus 10% FBS. Caspases inhibitor Z-VAD-FMK (C#G7231) and ROS scavenger N-acetylcyesteine amide (NAC) (C#A0737) were purchased from Sigma Aldrich.

To estimate cell viability, cells were cultured in 96-well plates at 2 × 10⁴ cells/well overnight, treated with atmospheric gas plasma for indicated time periods (5, 10 and 15 s), and incubated for 18–24 h. Cell viability was measured using CellTiter 96 Aqueous Non-Radioactive Cell proliferation.
(MTS) Assay (Promega, C#G5421) following the protocol provided by the manufacturer. In some experiments, the caspase inhibitor zVAD-FMK (50 μM) or NAC (3 mM) was added 1–2 h prior to atmospheric gas plasma treatment. NAC is an important antioxidant commonly used to identify and test ROS inducers and the role of ROS species in cell death. The antioxidative or free radical scavenging property of NAC stems from its ability to increase intracellular levels of an important natural antioxidant glutathione (for which NAC is a synthetic precursor), and through its reducing thiol-disulfide exchange activity.34 Pan-caspase inhibitor zVAD-FMK selectively inhibits caspases 3 and 7, and hence is commonly used to test whether observed cell death was attributed to caspase-dependent apoptosis. Assays were performed in triplicate.

To evaluate changes in cell gene expression as a result of atmospheric-pressure plasma treatment, a quantitative real-time polymerase chain reaction technique was used. Cells were cultured in 6-well plates at 5 × 10⁵ cells/well overnight, treated with atmospheric-pressure plasma for specified time periods, and incubated for 18–24 h. Following incubation, total cell RNA was extracted using Trizole reagent (Invitrogen, C#15596–026) and reverse transcribed into single-stranded cDNA following the procedure described previously.35 Quantitative relative gene expression was determined using SsoAdvanced SYBR Green SuperMix (Biorad, C#172–5265) with Roche Lightcycler 480 qPCR system. Data were analysed with LightCycler 480 software. The human p53 PCR array primers library (RealTimePCR.com, C#HTPS-1) was used to quantify relative gene expression of >90 genes involved in p53 signalling pathways. GAPDH, Actin-b, GUS-b, B2M, HPRT1, PPIA, and RPL13A were used as internal controls to normalize data. Relative quantification of gene expression after atmospheric gas plasma treatment was obtained by using the ΔCt method compared to fold change of untreated (He gas flow only) control cells.

For RNA interference and Western Blot analyses, cells grown to a density of ~70% confluence with antibiotic-free media were transfected with the TP73 siRNA (Santacruz, C#sc-43730) and negative control siRNA by lipofectamine (Invitrogen) according to the manufacturer’s instructions. After 24 h of siRNA transfection, cells were treated with atmospheric gas plasma. Cells were harvested and lysed in RIPA lysis and extraction buffer (C#89901), halted protease inhibitor cocktail (C#87786), and halted phosphatase inhibitor cocktail (C#78420) (thermoscientific) by incubating on ice for 30 min, as described previously.36 Protein concentration was determined by BCA protein assay kit (Thermoscientific, C#23227). After adding 2x SDS loading buffer, the samples were subjected to SDS-PAGE (Biorad, MP TGX 4%–20%, C#4561094). Protein was then transferred onto immunoblot polyvinylidene fluoride membrane (Millipore, Billerica, MA) and probed with the primary antibodies as specified and horseradish-conjugated secondary antibodies. The bonded proteins were visualized with a chemiluminescence detection kit (Biorad, Clarity western ECL substrate C#170–5060) using ImageQuant LAS400 (GE technology). GAPDH antibodies and TP73 (sc-9651) antibodies were purchased from Cell Signaling Technology and Santa Cruz Biotechnology, respectively.

III. RESULTS AND DISCUSSION

Cancer is a complex disorder that involves the alteration of intracellular homeostasis by a series of genetic mutations.37 In normal cells, there are a number of mechanisms to ensure that DNA mutations are not passed along to daughter cells, either through error correction (by arresting the cell cycle until the error is fixed) or by destroying the cell (through programmed cell death). Programmed cell-death is a general term used to denote death of a cell mediated by an intracellular program. Apoptosis is the process of programmed cell death, where intracellular biochemical events lead to characteristic cell changes, such as cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation, and eventually cell death. Other programmed cell-death types include autophagy, a catabolic process associated with autophagosomal-lysosomal degradation of bulk cytoplasmic contents, abnormal protein aggregates, and excess or damaged organelles; necroptosis, where caspase-independent inflammatory cell death (necrosis) is initiated in a programmed fashion; anoikis, where cell death is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix; excitotoxicity, where neuron cell death is induced by overstimulation with glutamate or related excitatory amino acids; ferroptosis, an iron-dependent form of cell death; Wallerian degeneration; and potentially others.

In cancer cells, these mechanisms are often compromised, with mutations passed along to subsequent generations. Plasma treatment provides the means to selectively induce apoptosis in cancer cells, potentially via activation of different tumour suppressors and inhibition of various oncogenes, without harming the healthy cells.22 This selectivity is highly desired, as most current cancer therapies tend to at least to some degree damage healthy cells. The apoptotic nature of atmospheric plasma-induced cell death is demonstrated using Annexin V/propidium iodide apoptosis assay that can differentiate between viable, apoptotic, or necrotic cells through differences in plasma membrane integrity and permeability.7,38

Apoptosis is a genetically regulated biological process with two pathways: the death-receptor-induced extrinsic pathway and the mitochondria-apoptosome-mediated intrinsic pathway. The Bel-2 family has a central role in controlling the mitochondrial pathway.39 Yan et al. have shown that Bel-2 family members are involved in atmospheric plasma-induced apoptosis. Atmospheric gas plasma treatment induced expression of Bax and inhibited Bel-2 expression determined the involvement of mitochondrial-mediated apoptotic pathway.40 In human embryonic kidney 293T cells, nitrogen plasmas activated a surrogate DNA damage signal transduction pathway, called the ataxia telangiectasia mutated (ATM)-checkpoint kinase 2 pathway; this effect suggests that the nitrogen plasma induced DNA double-strand breaks. Phosphorylation of H2AX and p53 was detected in the plasma-treated cells leading to apoptotic cell death.41,42 Human colorectal cancer cells...
treated with non-thermal He/O2 atmospheric plasmas experienced cell growth arrest and apoptosis. This treatment reduced the cell migration and invasion activities and also increased beta-catenin phosphorylation that might play a role at least in part in the plasma-induced anti-proliferative activity. It has been recently shown that the plasmas can specifically disable S-phase of the cell cycle in skin cancer cells. This effect was not observed in control cells demonstrating the plasma selectively induced apoptosis in cancer cells.

Plasma can often act synergistically in combination with other objects, such as nanoparticles and other drugs, by reducing the minimum inhibition concentration of nanoparticles or drug and hence reducing toxicity of the treatment. The synergy between plasma and nanoparticles for anticancer therapy can potentially be extended to the plasma-assisted fabrication of porous nanostructures that can be used to trap plasma-generated ROS/RNS species, to extend their half-life and ensure their passage deep into affected tissues, as well as facilitate the use of less chemically reactive materials in nanoparticle synthesis. Nanoparticles prepared in an atmospheric plasma jet can be both exposed to very high concentrations of ROS/RNS and simultaneously charged to facilitate their directional penetration into tissues. Mutual orientation of plasma ion fluxes and nanoarrays can further enable nanoparticles to target specific cellular sites. Another potential avenue involves pre-deposition of nanoparticles onto specific surface sites of cell membrane to form a cell-surface mask for spatially selective plasma treatment.

To ensure that plasma treatment is tuned for optimum selectivity and killing efficacy, it is critical to understand the biological processes that take place within the cell upon plasma exposure. The genetic manipulation of tumour cells to express or inhibit tumour-inducing or tumour-suppressing molecules provides a means to analyse cellular responses against tumourigenesis and anti-tumour therapeutics, such as plasma treatment. To explore possible signalling cascades involved in the plasma-induced cancer cell death, we quantified some of the key signalling genes involved in apoptosis.

In this work, we explored the effect of plasma treatment dose on the expression of tumour suppressor protein TP73, which, when overexpressed, is known to cause a growth arrest and/or apoptosis, and hence can potentially be targeted to enhance killing efficacy and selectivity of plasma treatment. TP73 belongs to the tumour protein p53 family. The p53 pathway responds to stresses that can disrupt the fidelity of DNA replication and cell division, with tumour protein p53 having many mechanisms of anticancer function, including apoptosis, genomic stability, and inhibition of angiogenesis. In a healthy cell, p53 is inactivated by its negative regulator, mdm2. This occurs through the direct binding of mdm2 to the N-terminal end of p53, which inhibits the transcriptional activation function of p53, and through E3 ubiquitin ligase activity of mdm2, which targets p53 for modification and subsequent degradation through the 26S proteasome. DNA damage or stress can initiate the physical dissociation of the p53 and mdm2 complex via a number of pathways. Activated p53 can induce cell cycle arrest (to enable DNA repair) or apoptosis.

A. Effect of plasma dose on tumour suppressor p53 cell signalling pathway

To explore possible signalling cascades involved in the plasma-induced cancer cell death, some of the key signalling genes (selected 85 genes as shown in Figure 2) involved in p53 signalling pathway were quantified by qPCR (presented and briefly described in Materials and Methods). As shown in Figure 2, BTG2, Casp2, Casp9, CCNB2, CDKN1A, IFNB1, PPKCA, PTEN, and TP73 were among the

![Graph A](image)

![Graph B](image)

**FIG. 2.** The results of qPCR analysis performed for the key 85 signalling genes involved in p53 signalling pathway. qPCR data were normalized; bars indicate the standard error of the mean, statistical analysis by t-test.
differentially expressed genes in melanoma Mel007 cells treated with atmospheric gas plasma.

Among them, the **tumour suppressor protein TP73 gene** was the most induced gene (up to 70 fold) compared to control untreated cells. Structurally, TP73 protein shares the same architecture with p53, with a significant identity in primary sequence, especially in DNA-binding domain and, to lesser extent in the transactivation domain which has pro-apoptotic effects. As a result, TP73 is able to transactivate several p53 responsive genes involved in the control of cell cycle arrest and apoptosis. Similar to p53, TP73 shows multiple distinct splicing isoforms (at least seven), with a resulting complicated array of functional effects.

These gene screening results demonstrate the importance of p53 family members, particularly high expression of TP73 in plasma-induced cancer cell death. This is consistent with previous reports that implicated TP73 in inducing apoptosis in cells with DNA damage via different signalling pathways. Up-regulation of TP73 (and apoptosis) has been observed in response to various DNA-damaging agents. The mechanisms by which TP73 can induce apoptosis in cancer cells involve induction of G1 cell growth, arrest and activation, the transcription of some endogenous p53 target genes, such as 14-3-3σ, mdm2, cyclin G, p21Waf1/Cip1, GADD45, ribosomal gene cluster (RGC), Bax, and insulin-like growth factor-binding protein 3 (IGF-BP3). Additionally, TP73 can induce apoptosis by activating PUMA/Bax signaling, where TP73 directly activates PUMA transactivation activity. The direct effect of PUMA on Bax promoter suggests a molecular link between TP73 and the mitochondrial apoptotic pathway.

To further study the effect of atmospheric gas plasma treatment on cancer cells and TP73 gene expression at RNA and protein level, melanoma Mel007 cells were treated for 5, 15, or 30 s (to vary the plasma dose). Figures 3(a) and 3(b) show the plasma increased TP73 RNA and protein expression in dose-dependent manner. Given that larger plasma doses (e.g., longer treatment times) are also associated with higher rate of cell death, the involvement of TP73 tumour suppressor protein in plasma-induced cancer cells death is likely.

**B. Plasma-induced melanoma cells death in TP73 dependent manner**

The specific effects of TP73 in plasma-induced cancer cell death were also explored. RNA interference (RNAi) specific to TP73 (TP73 siRNA) were used to knockdown the TP73 gene at RNA level. Gene knockdown, also known as gene silencing, describes the regulation of gene expression, where the expression of a certain gene is reduced but not completely eliminated. The reduced expression is achieved by interfering with either transcription (a process of copying a particular segment of DNA into RNA) or translation (a process of decoding of RNA by a ribosome to produce a specific polypeptide) stages of gene expression. Gene knockdown is commonly used in research to evaluate the significance of a particular protein in the observed intracellular processes.

As shown in Figure 4(a), TP73 siRNA treatment specifically inhibited TP73 protein expression in Mel007 cancer cells. To further confirm the role of TP73 in plasma-induced cancer cell death, siRNA-pre-treated Mel007 cells were subjected to plasma treatment. Cell viability assay showed that...
knockdown of TP73 by TP73 specific siRNA in cancer cells inhibited plasma-induced cytotoxic effects (Figure 4(b)).

The effect of TP73 silencing was similar to that of interfering with either TNF receptor 1 (using antagonist–neutralizing antibody vZAD) or intracellular ROS levels (using ROS scavenger N-acetyl-l-cysteine), or depletion of intracellular ASK1. In our previous work, we confirmed the oxidative stress-induced TNF–ASK1–JNK/p38–caspase-3/7 apoptotic pathway as one of the possible mechanisms for atmospheric gas plasma activation and regulation of apoptosis-signalling pathways in tumour cells. Briefly, extracellular ROS generated by plasma induce oxidative stress.

In response to this stress, the intrinsic mitochondrial pathway of apoptosis is induced, with increased expression of apoptosis signal molecules, primarily tumour necrosis factor-α (TNF-α), and activation of apoptosis signal-regulating kinase 1 (ASK1) pathway. ASK1 is a mitogen-activated protein kinase (MKK) that is activated by various stress-related stimuli, including oxidative stress, ROS, genotoxic agents, endoplasmic reticulum stress, and TNFs.

Intracellular ROS and ROS generated by TNFs disassociate a redox regulatory protein (thioredoxin, Trx) that inhibits the kinase activity of ASK1. The activated ASK1 phosphorylates and activates the downstream kinases MKK4/MKK7 and MKK6/MKK3. The initiation of these pathways increases downstream activity of c-Jun N-terminal (JNK) and p38 mitogen-activated protein kinases and stimulate activation of cysteine-dependent aspartate-specific proteases (caspase 3 and 7). These caspases proteolytically degrade a host of intracellular proteins, resulting in cellular shrinkage and DNA fragmentation, culminating in apoptosis.

Taken together, the results suggest that multiple signalling pathways mediate plasma-induced melanoma cells death, with tumour suppressor protein TP73 playing a notable role in plasma-induced apoptosis. The precise mechanisms by which plasma treatment induces TP73 up-regulation and how these events lead to melanoma cancer cell apoptosis need to be elucidated.

IV. CONCLUSION

This work represents an initial step in the studies of the effects of non-equilibrium, low-temperature atmospheric-pressure plasma on melanoma Mel007 cancer cells. One of the marked effects noted through the gene expression analysis of >90 genes involved in p53 signalling pathways is a clear up-regulation of tumour suppressor protein TP73. This study has revealed the effect of the plasma dose on the expression of TP73. Silencing of TP73 synthesis inhibited the anticancer effects of plasma. Importantly, anticancer efficacy of atmospheric-pressure plasma treatment is affected by the activity of the tumour suppressor protein TP73, which in turn can be plasma-dose dependent. Even though these results are obtained for the specific type of cancers, they nevertheless suggest that similar intracellular mechanisms may be deliberately induced by controlling the plasma exposure to halt or even reverse development of cancer in other cell types.

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