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Degradation of adhesion molecules of G361 melanoma cells by a non-thermal atmospheric pressure microplasma

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Abstract. Increased expression of integrins and focal adhesion kinase (FAK) is important for the survival, growth and metastasis of melanoma cells. Based on this well-established observation in oncology, we propose to use degradation of integrin and FAK proteins as a potential strategy for melanoma cancer therapy. A low-temperature radio-frequency atmospheric microplasma jet is used to study their effects on the adhesion molecules of G361 melanoma cells. Microplasma treatment is shown to (1) cause significant cell detachment from the bottom of microtiter plates coated with collagen, (2) induce the death of human melanoma cells, (3) inhibit the expression of integrin $\alpha_2$, integrin $\alpha_4$ and FAK on the cell surface and finally (4) change well-stretched actin filaments to a diffuse pattern. These results suggest that cold atmospheric pressure plasmas can strongly inhibit the adhesion of melanoma cells by reducing the activities of adhesion proteins such as integrins and FAK, key biomolecules that are known to be important in malignant transformation and acquisition of metastatic phenotypes.

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1. Introduction

Low-temperature plasmas generated at reduced gas pressures have been an indispensable technology for many industrial sectors, including microelectronic fabrication and display devices [1, 2]. Over the past 20 years, there has been growing interest in developing their counterparts at atmospheric pressure, particularly for biomedical applications such as bacterial inactivation [3, 4], wound healing [5], dental bleaching [6] and treatment of cancer cells [7, 8]. Among numerous low-temperature atmospheric pressure plasmas, including dielectric-barrier discharges [9] and radio-frequency (RF)-driven glow discharges [10], atmospheric pressure plasma jets feature particularly strongly in the aforementioned biomedical applications [11]–[15]. It has been known that they could be used to induce cell detachment and apoptosis (programmed cell death) [16, 17]. This is significant, because cell adhesion to the extracellular matrix (ECM) is important for many biological processes, such as embryogenesis [18], tumor invasion and metastasis [19]. Loss of cell adhesion may lead to a specific type of apoptosis known as ‘anoikis’ [20]. Thus, the ability of plasma to give rise to cell detachment and apoptosis has important implications for cancer treatment as well as other biomedical applications. It is, however, unknown what cellular processes may be triggered in plasma-enabled cell detachment. This study attempts to study whether low-temperature atmospheric plasmas compromise the physical link of cells to the ECM.

For this work, we consider melanoma cells that require adhesion to the ECM for their survival, growth and metastasis [21]. The physical link between ECM and the actin cytoskeleton is mainly mediated by cell-surface adhesion receptors. Integrins form one of the largest receptor groups and constitute a class of transmembrane glycoproteins that functionally link the cytoskeleton to the extracellular environment [22]. Their interaction with the actin cytoskeleton leads to integrins being redistributed on the cell surface and clustered onto specific structures known as focal adhesions, where the ends of the actin filaments are anchored to the membrane [23]. While stationary adult cells use the integrin–ECM interactions for anchorage, cancer cells appear to use these interactions for traction to migrate and possibly also as a
stimulus to proliferate. It is therefore of great importance that the integrin–ECM interaction is studied in plasma-facilitated cell detachment and apoptosis. At the tissue level, integrins also play key roles in regulating tumor growth, metastasis and angiogenesis. During melanoma development, the increased expression of integrins enables melanoma cells to convert from a stationary to a migratory and invasive phenotype [24]. The present study of integrin–ECM interactions in melanoma cells is likely to have direct implications to relevant tumor growth and metastasis processes.

To study whether and how low-temperature atmospheric plasmas may affect integrins and cell adhesion, it is important to consider focal adhesion kinase (FAK), a major signaling mediator whose activation requires both integrin attachment and cell spreading [25, 26]. FAK is a cytoplasmic tyrosine kinase that is responsible for mediating many cellular processes, including cell survival, migration and invasion. Unregulated increases in cell survival mechanisms, migration and invasion can enable tumors to metastasize to distant sites within the body. Therefore, FAK is often overexpressed in numerous cancers, including melanoma, breast, colon and thyroid cancers [27]–[29], whereas normal tissues express little detectable FAK. Moreover, inhibition of FAK expression causes apoptosis in several human tumor cell lines, including melanoma [30].

Inhibiting the expressions of integrins and FAK proteins may therefore be a good strategy in melanoma treatment. Significantly low-temperature atmospheric pressure microplasmas have recently been shown to destroy and degrade biomolecules such as proteins [31, 32], and so we hypothesize that atmospheric pressure microplasmas could also degrade integrins and FAK proteins in melanoma cells, compromising their interaction with ECM and ultimately leading to the induction of cell death. If this could be established, it would open the door to a new plasma-mediated strategy towards cancer therapy. Up to now, there has been no report on whether plasma treatment deactivates adhesive molecules such as integrin and FAK proteins and on whether plasma targeting of adhesion molecules could potentially be used in cancer therapy. This study takes a first step and investigates at a cellular level whether low-temperature atmospheric microplasmas inhibit the expression of integrins and FAK proteins at the focal adhesion sites of human melanoma G361 cells.

2. Materials and methods

2.1. Cold atmospheric pressure microplasma jet

A schematic diagram of the experimental setup is shown in figure 1(a). RF energy at 13.56 MHz was applied, via an impedance-matching network, to a tungsten wire of 0.4 mm diameter and the tungsten wire was enclosed within a concentric grounded cylinder having an inner diameter of 6 mm. The electrode unit was therefore of coaxial configuration. With an atmospheric pressure helium flow rate of 0.5–5 slm (standard liters per minute), a stable plasma was struck as shown in figure 1(b). The plasma diameter was around 1 mm, and its plume length was variable from less than a millimeter to a few millimeters, depending on the input RF power and the gas flow rate. The impedance-matching network was found to be critical in maximizing plasma density. The RF generator was designed to operate at up to 10 W of input power with a sweeping step of 0.1 W. The gas temperature at the tip of the microplasma jet was measured using a temperature strip at the position of the sample and confirmed with rotational temperature measured from the nitrogen monopositive ion N_{2}^{+} first negative system (B^{2} \Sigma_{u}^{+}, v = 0 \rightarrow X^{2} \Sigma_{g}^{+}, v' = 0, 388–392 nm).
2.2. Optical emission spectroscopy

The optical emission spectrum of the atmospheric pressure microplasma was measured with a spectrophotometer (SpectraPro 300i, ARC, 15 Discovery Way, Acton, MA 01720, USA) from the light detected with an intensified charge-coupled device (PI-MAX2, Princeton Instruments, 3660 Quakerbridge Road, Trenton, NJ 08619, USA) in shutter mode, with integration performed over 10 ms and at a gain of 1.0. The grating used was 600 grooves mm$^{-1}$ when used to identify reactive plasma species, and 2400 grooves mm$^{-1}$ when used to measure rotational temperature.

2.3. Cell culture

G361 melanoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM HEPES, 100 $\mu$g ml$^{-1}$ penicillin/streptomycin, 4 mM L-glutamine and 10% fetal bovine serum at 37 °C in a 5% CO$_2$ humidified-air incubator.

2.4. Cell viability assay

The viability of cultured cells was estimated by sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay. XTT solution was made by completely dissolving XTT in hot media at 1 mg ml$^{-1}$. This was most easily accomplished by heating a tube containing medium in a 60 °C water bath. Menadione (MEN) was made up fresh each day as a 100 mM solution in dimethyl sulfoxide, and was added to the XTT solution immediately before use. Twenty microliters of XTT/MEN was added to each 100 $\mu$l culture. After an additional 4 h of incubation at 37 °C, the optical density of the wells was determined using a multiwell reader (Quart, Bio-Tek, Winooski, USA) at a test wavelength of 450 nm and a reference wavelength of 650 nm. Each plate contained appropriate blank control wells containing media, XTT and MEN, but no cells.

2.5. Inhibition of adhesion assay

Flat-bottomed wells in microtiter plates were coated overnight at 4 °C with 100 $\mu$l of collagen type I (50 $\mu$g ml$^{-1}$). G361 cells (4 × 10$^5$) in 100 $\mu$l of DMEM were plated in triplicate on each
coated well and incubated for 24 h at 37 °C. After treating the cells with the RF microplasma, unbound cells were removed by two gentle washes with phosphate buffered saline (PBS). Attached cells were quantified by the XTT assay, and absorbance was measured at 450 nm. The numbers of adhering cells were calculated by reference to the standard curve for G361 cell suspensions (1 × 10^3/well to 4 × 10^5/well).

2.6. Fluorescence staining

After being treated by the RF microplasma, cells were fixed in 4% paraformaldehyde for 10 min. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min. For filamentous actin (F-actin) staining, cells on coverslips were labeled with FITC-phalloidin at room temperature for 30 min, and then rinsed in PBS three times for 5 min each time and mounted onto glass slides with 50% glycerol in PBS. For antibody staining, cells on coverslips were incubated with anti-integrin α2, anti-integrin α4 and anti-FAK antibodies for 1 h. Cells were washed thoroughly and then incubated with goat FITC-anti-mouse secondary antibody for 1 h. Cells were washed thoroughly in PBS three times for 10 min each. Coverslips were mounted onto glass slides with 50% glycerol in PBS and observed under a fluorescence microscope (Axioskop, Zeiss, Germany).

2.7. Western blot analysis

Cells treated with the microplasma were washed twice with ice-cold PBS, re-suspended in 200 μl of ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% TritonX-100, 2 mM PMSF, 2 μg ml⁻¹ aprotinin and 2 g ml⁻¹ leupeptin] and incubated at 4 °C for 30 min. The lysates were centrifuged at 14 000 revolutions/min for 15 min at 4 °C. Protein concentrations of cell lysates were determined with the Bradford protein assay (Bio-Rad, Richmond, CA, USA) and 50 μg of protein was loaded onto 7.5–15% SDS–PAGE. The gels were transferred to a nitrocellulose membrane and reacted with anti-FAK antibody. Immunostaining with antibodies was performed using enhanced chemiluminescence substrate (Supersignal West Pico) and detected with LAS-3000PLUS (Fuji Photo Film Company, Kanagawa, Japan).

2.8. Plasma treatment of G361 melanoma cells

The atmospheric pressure microplasma jet shown in figure 1 was applied to treat G361 melanoma cells in a petri dish. Nominal conditions were (1) an input RF power of 4 W, (2) a helium flow rate of 2 slm and (3) the plasma-housing unit positioned 3 mm from the surface of the human melanoma G361 cells.

3. Results and discussions

Plasma conditions were monitored using electrical measurement and optical emission spectroscopic measurement. In general, the electric circuit incorporating the impedance-matching network was well controlled. This helped to achieve a good reproducibility of the findings of our cellular experiments. Reflected RF power from the microplasma was found to be low in most cases and so the input RF power may be regarded as approximately proportional to the dissipated power in the microplasma. Gas temperature was measured with a temperature strip, and was in general consistent with rotational temperature measured from the N₂ band.
Figure 2. Gas flow rate dependence of gas temperature at the sample point at different input RF powers.

of 388–392 nm. Figure 2 suggests that gas temperature at the sample point was below 30°C as long as the input RF power was maintained at no more than 6 W. For all cell experiments reported here, the input RF was fixed at 4 W and the treatment time was nominally at 15 s. Therefore, cells under microplasma treatment were unlikely to experience a gas temperature more than 30°C and all their responses were likely to be non-thermal. It is worth mentioning that RF-driven atmospheric plasmas are often hotter than their low-frequency counterparts [33], particularly when driven at large RF power. For our experiments, the input RF power was low and the gas flow rate was sufficiently high, both helpful in cooling down the gas effluent. The very short plasma treatment time of 15 s was an additional factor to ensure that cells experienced non-thermal effects of the RF microplasma jet.

Figure 3 shows optical emission spectra at a fixed input RF power of 4 W and with different gas flow rates. Similar to other studies of atmospheric pressure plasma jets in helium, the optical emission spectra are dominated by helium species (particularly around 706 nm) as well as oxygen and nitrogen lines. The latter stemmed from the mixture of helium flow with ambient air. Of particular interest is the atomic oxygen line at 777 nm, as oxygen atoms have been shown to play a major, if not dominating, role in inactivation of both bacteria [4, 34] and biomolecules [31, 32]. The emission spectra of figure 3 are seen to vary markedly with the gas flow rate, due to helium gas displacing the air, thus changing the composition of the gas mixture at the tungsten tip [35, 36]. For example, simulation of gas fluid dynamics using a computational fluid dynamics code CFD-ACE+ [37] indicates that the molar fraction of air at the needle tip is lower than 1% when the helium velocity is larger than 0.8 m s$^{-1}$. A linear gas velocity of 0.8 m s$^{-1}$ is expected when the electrode unit of figure 1 is flushed with a gas flow rate of more than 0.5 slm. This suggests that the oxygen admixture in helium flow at the tungsten tip was likely to be less than 0.2%. However, the oxygen admixture along the length of the plasma plume may be slightly larger because a much increased radial diffusion of the working gas after exiting the electrode unit would reduce the effect of gas displacement. As a rough estimate, the oxygen content in background helium gas may vary between 0.2% and 2% in our experiments. This is the range within which bactericidal effects of low-temperature atmospheric plasma jets were found to be most significant against both microorganisms and biomolecules [4, 31, 32, 34].
Figure 3. Optical emission spectroscopy at 4 W of input RF power and different gas flow rates from 0.7 to 5 l min\(^{-1}\) (lpm).

Cell migration, homing and settlement during tissue formation, repair, tumor invasion and metastasis are guided by a complex set of adhesive interactions between cells and the ECM [18, 19]. These interactions are mediated by cell-surface adhesion receptors, of which the integrins are known to be one of the largest groups. The cytoplasmic tails of integrin \(\alpha\) and \(\beta\) subunits connect the ECM to the actin cytoskeleton and trigger intracellular signaling by recruiting many structural and adaptor proteins leading to intracellular kinase cascades. In this study, G361 cells were plated on collagen-coated wells of a microtiter plate and incubated for 24 h. Treatment of G361 cells using the RF atmospheric microplasma was found to significantly decrease their adhesion to collagen in a time-dependent manner as shown in figure 4(a). Over the same time course, figure 4(b) suggests that the viability of plasma-treated G361 cells, as estimated by the XTT assay, also decreases monotonically. However, it is interesting to note that after 15 s of the microplasma treatment, about 90% G361 cells seem to have lost their adhesion capability while the viability of the cell population appears to remain at approximately 20%. This suggests that the absence of attached G361 cells may be a consequence of not only cell death but also considerable loss of certain cell functions, particularly adhesion to the ECM.

It is known that actin or other components of the cytoskeleton are organized through distinct steps during cell adhesion. In the early phases, cells attach and begin to spread on the substrate, organizing actin into peripheral filopodia. After the adhesive process is completed, cells are stretched by the tensile forces of actin stress fibers to acquire a spindle shape. Stress fibers are anchored to the membrane at the focal adhesion structure. Figure 4(c) shows that many untreated G361 cells take a spindle shape and are likely to have achieved strong adhesion whereas other G361 cells exhibit a more rounded shape, indicating an incomplete adhesive
Figure 4. 15 s treatment of RF atmospheric microplasma inhibited (a) the adhesion and (b) viability of G361 cells. It also changed the cell morphology from (c) a spindle shape to (d) a more rounded shape.

process. Following microplasma treatment, figure 4(d) shows that many treated G361 cells change to a more round shape and even the spindle-shaped cells tend to become swollen with less defined border lines. These suggest a plasma-mediated compromise of cell functions. In fact, within a few seconds of plasma treatment when there were many viable G361 cells (see figure 4(b)), we observed that the usual spindle shape of live cells was changed to the more rounded shape. This indicates that loss of cell adhesion to ECM was already very significant prior to cell death, and is consistent with our hypothesis that plasma-facilitated cell death may be related to integrin–ECM interactions.

Plasma-mediated change of human melanoma cells from their normal states could be seen in the responses of adhesion proteins, particularly those of F-actin, a major cytoskeletal protein involved in cell motility [38]. To see this clearly, we stained G361 melanoma cells with the F-actin-specific probe, FITC-phalloidin, after 15 s of microplasma treatment. As shown in figure 5, fluorescent-labeled F-actin bundles appear at the edge of the spreading cells (untreated) whereas plasma-treated cells seem to attain a diffusive pattern of F-actin. This diffusive pattern lacks the distinct direction of spreading of control cells, and may be regarded as an indication of compromised or degraded functions of F-actin proteins.

To provide further support to the observation of plasma-mediated compromise of cell morphology and adhesion proteins in figures 4 and 5, we consider possible plasma damage to integrin proteins. It is known that integrins regulate the anchorage-independent growth
Figure 5. Degradation of well-stretched F-actin into a diffuse pattern by 15 s atmospheric microplasma treatment: (a) before and (b) after plasma treatment. Arrows indicate F-actin.

and invasive growth of tumors. In particular, they prevent apoptosis of invading cells and increase tumor growth [38, 39]. Many studies have reported that the expression of integrins changes frequently during malignant transformation. Furthermore, up-regulation of expression of integrins is associated with the acquisition of a more metastatic phenotype [40]. It has been reported that the expression levels of integrins αv, α2, α3 and α4 are increased in primary and metastatic melanomas [41, 42]. Therefore, the expression level of integrins αv, α2, α3 and α4 provides a direct indication of whether plasma treatment may influence directly malignant transformation and acquisition of a more metastatic phenotype. To this end, we performed an immunocytochemistry assay for integrins α2 and α4 after the G361 melanoma cells were treated with the RF atmospheric microplasma. Figure 6 shows the results of immunofluorescence microscopy for integrins α2 and α4 using each integrin-specific antibody. The shape of control cells is expanded with filopodia, with integrins α2 and α4 being located at the periphery. Treatment with the RF atmospheric microplasma resulted in a distinct change in cell shape from a spindle type to a more rounded type, with the expression of integrin disappearing on the cell surface. It should be emphasized that integrins α2 and α4 were stained with antibodies and the considerable reduction of their fluorescence intensity after microplasma treatment supports strongly the idea that these integrins are degraded. These results suggest that the RF atmospheric microplasma is likely to have degraded integrins α2 and α4 and hence inhibited their activity in our experiments. Since the plasma condition used in this experiment (4 W for 15 s) would still support some viable G361 cells (see figure 4(b)), it is unlikely that the observed integrin degradation resulted from blanket plasma damage to the whole cells. Instead, plasma treatment may have specifically degraded adhesion proteins of melanoma cells to ECM.

Finally we considered FAK proteins, because their up-regulation and uncontrolled signaling are known to be responsible for the promotion of malignant phenotypic characteristics and the resistance to chemotherapy. Figure 7 shows the results of immunocytochemistry and Western blot analysis. While FAK appears in lamellipodial edges as well as in outlining areas of control cells without plasma treatment, little expression of FAK is evident in the cells treated with the RF atmospheric microplasma. Similarly, Western blot analysis indicates significant reduction at the 125 kDa band. These results are direct evidence of our hypothesis that plasma treatment inhibits the expression of FAK. It should be mentioned that direct FAK targeting is known to inhibit the malignant phenotype of cancer cells [43]. Together with consistent
Figure 6. Inhibiting effects of RF atmospheric pressure helium microplasma (4 W, 15 s) on the expressions of integrins $\alpha_2$ and $\alpha_4$ in G361 melanoma cells, with (a) and (b) control cells ($\alpha_2$ and $\alpha_4$ marked with white and yellow arrows, respectively), and (c) and (d) plasma-treated cells.

Figure 7. Inhibitory effects of atmospheric pressure helium plasma (4 W, 15 s) on the expression of FAK in G361 melanoma cells as shown (a) before plasma treatment and (b) after plasma treatment, both illustrated with immunocytochemistry (yellow arrow marking FAK), and (c) with Western blot analysis.

results for integrins $\alpha_2$ and $\alpha_4$ in figure 6, it is clear that a short treatment of 15 s with the RF atmospheric microplasma operated at a low input power of 4 W is capable of inhibiting the expression of integrins $\alpha_2$ and $\alpha_4$ and FAK. Given the significance of integrins $\alpha_2$ and $\alpha_4$ in malignant transformation and that of $\alpha_2$, $\alpha_4$ and FAK in acquisition of a more metastatic phenotype, the results in figures 6 and 7 are very encouraging.

The results represented in this study are motivated by the need for a basic understanding of how cold atmospheric plasmas interact with cancer cells. While it is conceivable that plasma inactivation of adhesion proteins may be used to control the migration of cancer cells and their...
acquisition of a metastatic phenotype, more studies are needed before an effective practical implementation may be reached. An important issue here is possible UV damage, particularly around 260 nm in the UVC band (100–280 nm), that may lead to the formation of pyrimidine dimers that can cause mutagenesis and cell death during DNA replication [44]. If the results reported here were accompanied by considerable UVC triggered mutagenesis or due to UVC triggered cell death, the viability of RF atmospheric microplasma as part of a cancer therapy would become questionable. According to relevant EU guidelines, the maximum allowed UVC dose rate is 30 $\mu$W cm$^{-2}$ [45]. To investigate this, we measured the emission spectrum of the RF atmospheric plasma jet for the band of 200–900 nm with its emission intensity absolutely calibrated for the 350–850 nm band, under the condition of 4 W RF power and 2 slm helium flow. The strongest emission was found to be the helium line at 706 nm with its emission intensity well below 1 $\mu$W cm$^{-2}$, and its emission intensity in the 250–300 nm range was only a small fraction of 1 $\mu$W cm$^{-2}$. With a smaller helium flow rate, emission from the N$_2$ (C-B) band became proportionally stronger (see figure 3(c)) but the absolute emission intensity remained well below 1 $\mu$W cm$^{-2}$. This is not dissimilar to the emission of similar RF atmospheric helium plasma jets [46], and suggests that the UVC emission intensity is likely to be well below the maximum allowed dose rate of 30 $\mu$W cm$^{-2}$. Hence UVC irradiation of the RF atmospheric plasma jet is unlikely to cause mutagenesis and the cell death shown in figure 4 is likely to be due to plasma treatment rather than UV. It is useful to remark that UVC damage is better characterized in terms of dose than dose rate and that the maximum allowed dose rate above is arrived at in a general context of sunburn [45]. The timescale of the sunburn is much greater than the timescale of atmospheric plasma treatment considered here. Therefore, UVC radiation associated to the RF atmospheric plasma jet considered is most likely to be below the relevant safety threshold and the deactivation effects of adhesion proteins shown in figures 4–6 are triggered by reactive plasma species, particularly reactive oxygen species.

4. Conclusion

An RF atmospheric microplasma jet was used for the treatment of human melanoma cells. By controlling the input RF power and the flow rate of the background helium gas, the gas temperature was found to be below 30 °C and the plasma effects were predominantly non-thermal. Optical emission spectra indicated the presence of abundant oxygen species, including oxygen atoms, which are known to inactivate both microorganisms and proteins.

Short treatment of 15 s with the RF atmospheric microplasma operated at 4 W of input power was found to change the morphology of G361 melanoma cells from their usual spindle shape to a more rounded shape when there were still many viable cells. A similar shape change also occurred for one adhesion protein (filamentous actin). Furthermore, the same plasma treatment was shown to inhibit the expressions of integrin $\alpha_2$, integrin $\alpha_4$ and FAK, which are known to be important in malignant transformation and acquisition of metastatic phenotypes. Finally, microplasma treatment was found to cause a marked reduction in actin filaments and to induce a diffusive pattern in G361 melanoma cells that lacks directionality of the usual spindle form. These results indicate that low-temperature atmospheric microplasma could strongly inhibit the proliferation of melanoma cells through potentially specific degradation to focal adhesion molecules (e.g. a plasma-mediated molecular process) and hence a new route towards melanoma cancer therapy. Much needs to be studied further both at a cellular level and at a tissue level, some of which will be reported in future correspondences.
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