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Air plasma coupled with antibody-conjugated nanoparticles: a new weapon against cancer

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Abstract
Ambient air plasmas have been known to kill cancer cells. To enhance selectivity we have used antibody-conjugated nanoparticles. We achieved five times enhancement of melanoma cell death over the case of the plasma alone by using an air plasma with gold nanoparticles bound to anti-FAK antibodies. Our results show that this new interdisciplinary technique has enormous potential for use as a complement to conventional therapies.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

In our difficult war [1, 2] against cancer in the past few decades, a drastically novel therapeutic approach has emerged. This uses antibody-conjugated nanoparticles with plasma. Promising progress in the plasma and pulsed power community has enabled the generation of room-temperature atmospheric-pressure plasmas and a pulsed electric field suitable for biomedical applications [3–5]. The non-thermal air plasma has shown its effectiveness in killing cancer cells [6]. However, there is no selectivity between normal and cancer cells. Recent progress in nanotechnology has led to biomedical applications such as diagnostic tools for early cancer detection and drug delivery through conjugated-gold nanoparticles [7–9]. We have used 30 nm gold nanoparticles and antibody conjugation to selectively enhance the therapeutic effects of the plasma. Gold nanoparticles are frequently used in bio-applications due to their unique characteristics. First, gold nanoparticles are relatively easily prepared with controlled size, morphology and surface properties. Second, gold nanoparticles' strong scattering and absorption of light due to the excitation of the resonant surface plasmon make them suitable for diagnostic applications and laser photo-thermal therapy [10]. The important aspects in dealing with gold nanoparticles in bio-applications are safety and biocompatibility (gold nanoparticles are already approved by the US Food and Drug Administration.) We discuss the toxicity of gold nanoparticles under cell proliferation in the following section.

2. Methods and materials

Figure 1(*a*) shows a schematic of the experimental setup used to generate the plasma. Two electrodes covered with a dielectric material were connected to each other to generate a plasma. One electrode was connected to a low frequency (22 kHz), high voltage (5 kV) sinusoidal source and the other

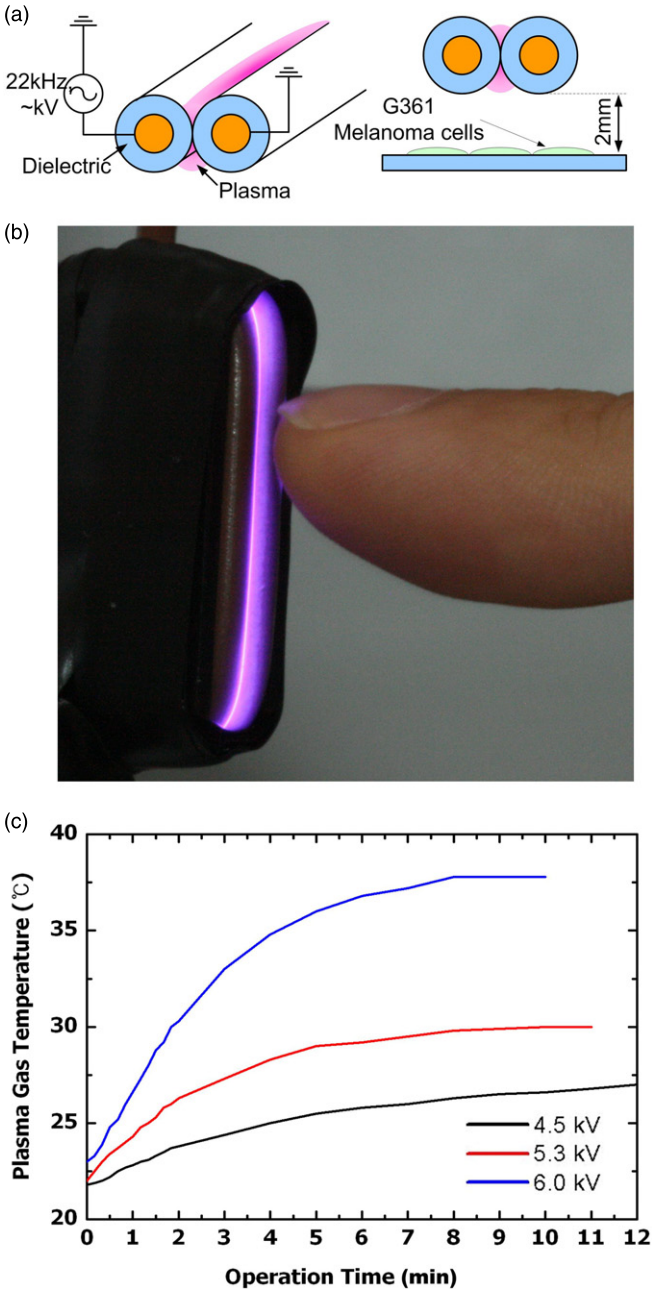

Figure 1. Plasma device characteristics. (*a*) Schematic diagram of the plasma device and the experimental setup. The cells were cultured on the cover glass for plasma treatment. (*b*) Actual air plasma source. (*c*) The evolution of the sample temperature as recorded with an optical thermometer.

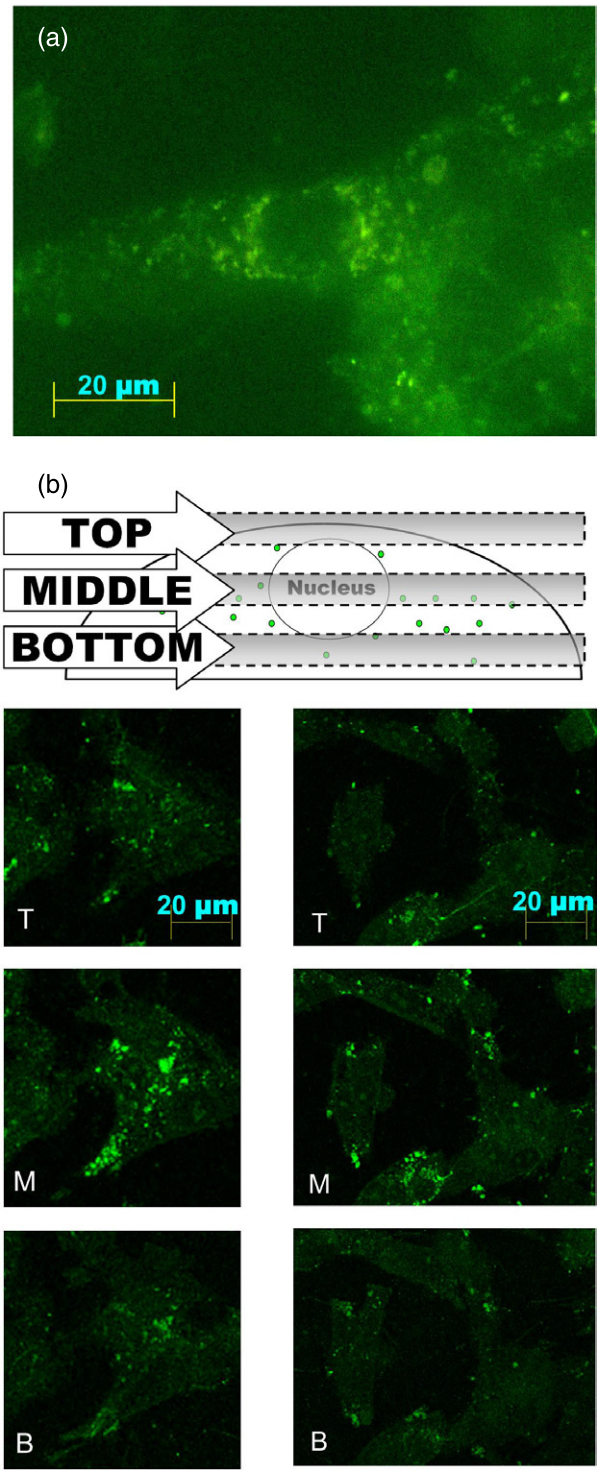

Figure 2. Localization of gold nanoparticles in G361 cells. (*a*) Uptake of colloidal gold nanoparticles (1000×). The bright (green) spots represent the gold nanoparticles absorbed by G361 cells. (*b*) A proposed model of cells absorbing gold nanoparticles and actual images (400×) from a confocal laser scanning microscope. The gold nanoparticles were observed mainly in the middle of the cytoplasm. T, M and B represent top, middle and bottom, respectively. (Colour online.)

electrode was grounded. This setup operates in ambient air without any gas tanks and a vacuum system. A picture of the actual plasma source is shown in figure 1(*b*). Despite the high voltage used to operate the device, the dielectric material covering the electrodes limits the voltage and current across the plasma itself, making it safe to touch the plasma during device operation (figure 1(*b*)). In this experiment, G361 human melanoma skin cancer cells were placed 2 mm from the plasma source and exposed to 40 s plasma treatment. The air plasma source used in this work does not induce thermal stresses to the sample as the temperature remains below 38 °C even after a 10 min exposure (×15 the duration of a normal treatment) to a plasma driven at a 10% overvoltage (figure 1(*c*)).

The gold nanoparticles conjugated with antibodies were prepared as follows. An aqueous solution of

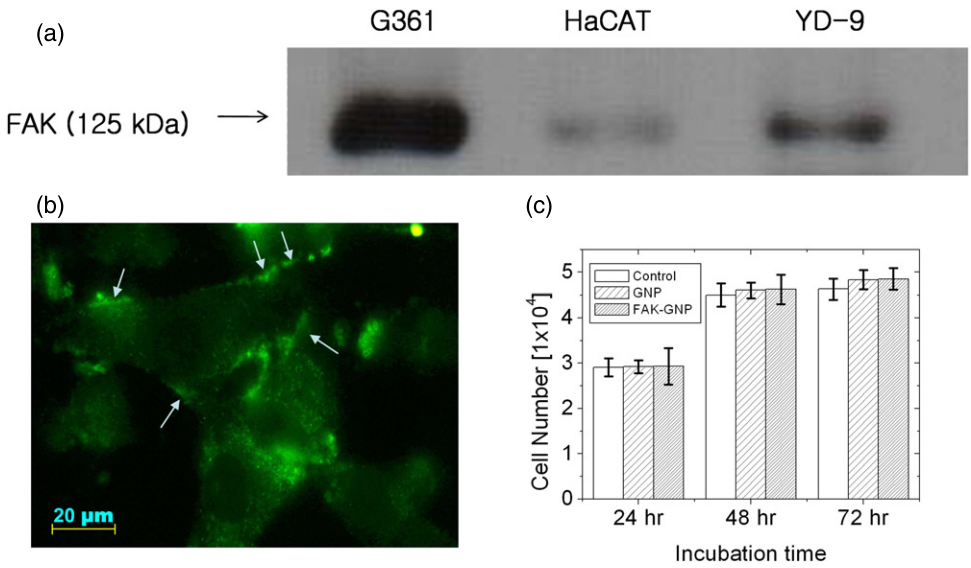

Figure 3. Localization of FAK-GNPs in G361 cells. (*a*) Overexpression of the FAK protein in G361 melanoma cells compared with normal keratinocyte (HaCAT) and oral squamous carcinoma (YD-9) as shown by a Western blot assay. (*b*) Uptake of FAK-GNPs (1000×). The FAK-GNPs specifically bind to FAK and generates fluorescence around the cell membrane (arrow). (*c*) The viability measured by XTT over a 72 h incubation. There is no difference between control and experimental groups.

11-mercaptoundecanoic acid (MUA) (0.1 mg ml^{-1}) was added to the colloidal gold suspension and incubated overnight. MUA-modified gold nanoparticles were reacted with a mixture of 1 mM N-hydroxysuccinimide (NHS) and 1 mM N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC) solution for 20 min. NHS-terminated gold nanoparticles were incubated with a 250 μg ml^{-1} FITC conjugated secondary antibody and an anti-phospho-focal adhesion kinase (FAK) antibody in PBS buffer (1 mM, pH 7.0) for >8 h.

G361 cells were grown in Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES, 100 μg ml^{-1} penicillin/streptomycin, 4 mM L-glutamine and 10% fetal bovine serum at 37 °C in a 5% CO_2 humidified-air incubator.

Cells were treated with anti-FAK antibody-conjugated gold nanoparticles (FAK-GNPs) and then kept in 4% paraformaldehyde for 10 min. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and then incubated with goat FITC-anti-mouse secondary antibody for 1 h. The samples were observed under a fluorescence microscope (Axioskop, Zeiss, Germany).

The XTT solution for the XTT assay was made by completely dissolving XTT in hot media at 1 mg ml^{-1}. Menadione (MEN) was made fresh each day as a 100 mM solution in dimethyl sulfoxide and was added to the XTT solution immediately before use. Twenty microlitres of XTT/MEN was added to each 100 μl culture. After an additional 4 h of incubation at 37 °C, the optical density of the wells was determined using a multiwell reader (Quant, Bio-Tek, Winooski, USA) at a test wavelength of 450 nm and a reference wavelength of 650 nm.

Cells were lysed in ice-cold lysis buffer (300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% TritonX-100, 2 mM PMSF, 2 μl ml^{-1} aprotinin and 2 μl ml^{-1} leupeptin) and incubated at 4 °C for 30 min. 50 μg of proteins were loaded onto 7.5% SDS/PAGE. The gels were transferred to nitrocellulose membrane and reacted with an anti-FAK antibody. Immunostaining was performed using a SuperSignal West Pico enhanced chemiluminescence substrate and detected with LAS-3000PLUS (Fuji Photo Film Company, Kanagawa, Japan).

3. Results and discussion

Figure 2(*a*) shows the uptake of the gold nanoparticles by G361 melanoma cells. Gold nanoparticles were conjugated with a fluorescein conjugated, affinity purified secondary antibody and the results were examined by fluorescence microscopy. The bright (green) spots in figure 2(*a*) represent the gold nanoparticles absorbed by G361 cells over 24 h. The gold nanoparticles were distributed throughout the spindle-shaped cytoplasm, but not in the nucleus when they were observed from the top of the cells. The observed figures agree well with the light scattered image of malignant oral epithelial cells (HOC 313 clone 8) after absorbing 35 nm gold nanoparticles [11]. The location of gold nanoparticles was verified using a confocal laser scanning microscope to capture the fluorescence images scanning from the bottom to the top of the cells. The gold nanoparticles were distributed in the middle of the cytoplasm, and in many cases they were located around the nucleus (figure 2(*b*)).

The increased expression of FAK is crucial for the survival, growth and metastasis of melanoma cells [12, 13]. Furthermore, FAK is often overexpressed in numerous cancers, including melanoma, whereas normal tissues express little detectable FAK [14]. In our preliminary studies, the expression of FAK in G361 melanoma cells was higher than in the nonmalignant epithelial cell line (figure 3(*a*)). Degradation of FAK, therefore, may become a good strategy for efficient and selective melanoma therapy. This possibility was explored by conjugating a mouse anti-phospho-FAK (Y397)

monoclonal antibody to gold nanoparticles, according to the method described in [15, 16]. An immunocytochemistry assay demonstrated the uptake of FAK-GNPs into G361 cells (figure 3(*b*)). Once FAK-GNPs translocated across the cell membrane, they bound to FAK. As a result nanoparticles appear in the lamellipodial edges and in the outlining areas of the cells, where FAK is most abundant.

Before the plasma stimulation, the toxicity of gold nanoparticles and FAK-GNPs was determined by measuring cell proliferation. Gold nanoparticles with diameters in the 3–5 nm range are known to inhibit the proliferation of multiple myeloma cells [17]. The adopted 30 nm gold nanoparticles, however, do not produce any observable cytotoxic effect on the proliferation of G361 cells, as measured by the XTT assay after incubation for 72 h (figure 3(*c*)).

FAK was more expressed in G361 cells than in normal HaCAT cells and non-metastatic YD-9 cells. Moreover, gold nanoparticles have been shown to improve the efficacy of plasma treatment. The FAK-GNPs are expected to be not only more lethal but also more selective against G361 melanoma cells. To prove this hypothesis, three cell groups were prepared: (1) cells cultured in only media (figure 4(*b*)); (2) cells cultured in media containing gold nanoparticles (figure 4(*c*)) and (3) cells cultured in media containing FAK-GNPs (figures 4(*d*)). When the three groups of cells were irradiated by the plasma, the cell death rates were 14%, 36% and 74%, respectively (figure 4(*f*)). After the FAK-GNPs bind to FAK proteins specifically, irradiation of plasma stimulated gold nanoparticles caused deactivation of FAK, thereby drastically increasing the death rate to 74%. Considering that FAK is a major signalling mediator, it is very important to know which signal leads to cell death. However, that is unclear at present. Nonetheless, these results show that we can achieve a precise attack against cancer cells using plasma and functionalized conjugates made of gold nanoparticles and cancer specific antibodies and suggest that FAK is directly linked to cell survival as well as being a signalling mediator.

It is worth noting the cell morphology in the case of FAK-GNPs. When the FAK protein is attacked specifically, the typical spindle-type cell morphology was maintained, even in dead cells (figures 4(*d*) and (*e*)). This is a puzzling observation that has not been reported before. When cells die from plasma irradiation, they undergo anoikis, a form of apoptosis in which cells detach from the surrounding extracellular matrix. FAK-GNPs, however, did not induce anoikis.

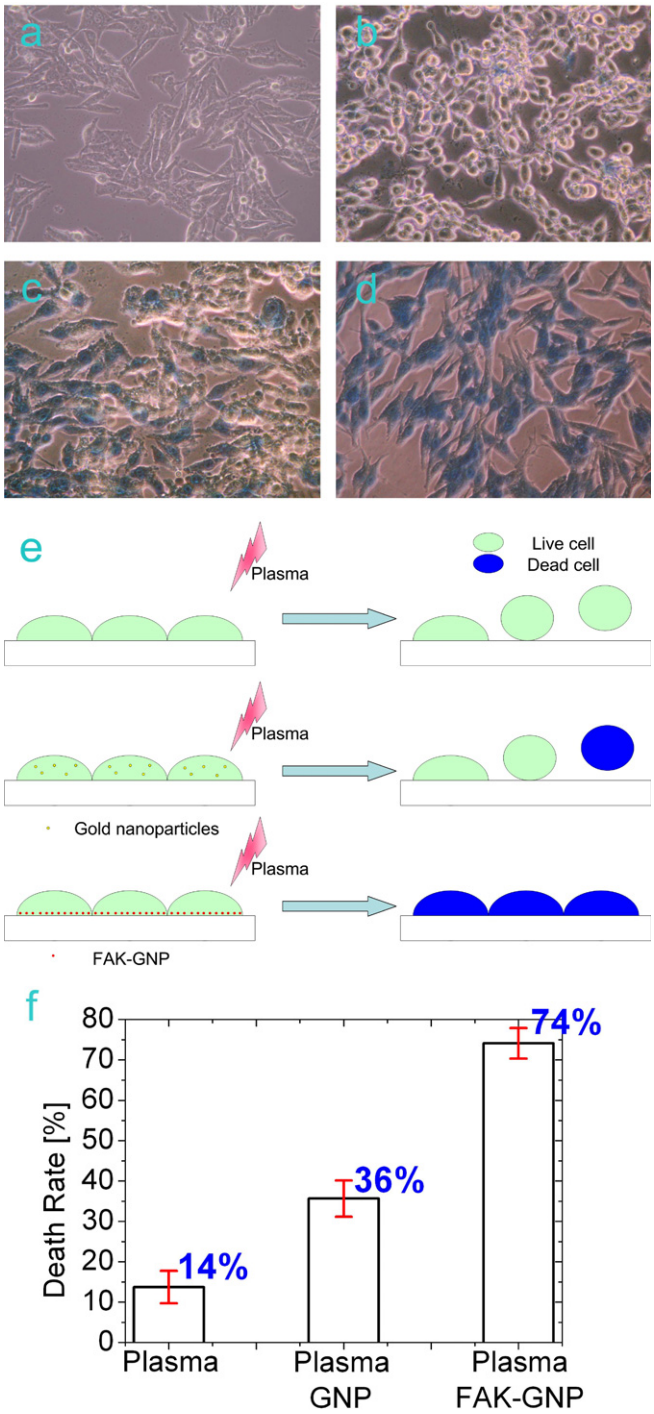

Figure 4. Contribution of FAK-GNPs to dramatic G361 cell death with plasma irradiation. For all the cases, the same dose of plasma (5.3 kV) was used and dead cells are stained in dark (blue) colour. (*a*) Microscope image without plasma treatment (200×). (*b*) Microscope image after the plasma treatment without gold nanoparticles (200×). (*c*) Microscope image after the plasma treatment with gold nanoparticles (200×). (*d*) Microscope image after the plasma treatment with FAK-GNPs (200×). (*e*) Plasma-induced cell death. (*f*) Comparison of the cell death rate which is significantly increased in the case of anti-FAK antibody-conjugated gold nanoparticles. (Colour online.)

4. Conclusion

Taken together, our study demonstrates that non-thermal plasmas can stimulate gold nanoparticles located inside cells to cause cell death even with a low-dose plasma treatment. This air plasma is coupled with FAK-GNPs, resulting in a 5× increase in cell death over the case with the plasma alone. This research opens the door to a new paradigm where the non-thermal plasma and antibody conjugated-gold nanoparticles team up to create a powerful weapon against cancer.

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