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Use of Proteomics to Investigate Plasma-Cell Interactions

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ABSTRACT: The understanding of basic mechanisms of plasma effects on living cells is one of the main preconditions to develop systematically innovative therapy options in the new and emerging field of plasma medicine. In this study, proteomics have been used for the first time to analyze the influences of physical plasma on vital constituents of mammalian cells. Treatment of human keratinocytes (HaCaT cells) by an atmospheric pressure argon plasma jet resulted in changes of charges of several cell proteins, but not in mass changes. These first results indicate plasma-induced reactions of functional groups or ligands, but no fragmentation, degradation, or complexation of cell proteins. Hereby, the importance to examine internal cellular changes caused by plasma treatment to elucidate the influence of plasma on the metabolism of human cells next to morphological changes, cell performance, and cell viability could be demonstrated. Starting from now, proteomics will become a useful tool for basic research in the field of plasma medicine.

KEY WORDS: plasma medicine, proteomics, two-dimensional gel electrophoresis, cellular proteome, atmospheric pressure plasma

I. INTRODUCTION

During the last years, increasing research interest to use physical plasma for biological and medical applications has led to the development of the new research field of plasma medicine as an intersection of plasma physics and life sciences.[1,2] However, to open up innovative options in medical therapies, basic understanding of the mechanisms of how physical plasma interacts with living system on the cellular level is essential.

Proteins are the main components of the physiological metabolic pathways of cells. The proteome is the entirety of proteins expressed by a living system, e.g., a cell, primarily dependent on the genome but influenced by several internal and external factors. Consequently, the cellular proteome is an indicator for the state of a particular cell type, at defined conditions at any given time. Proteomics is the use of techniques to define the protein pattern of a living system, e.g., a cell.[3] Because the proteome is in a constant balance of new synthesis and degradation of proteins, it undergoes permanent alteration in its composition, depending on environmental and internal conditions of the cells. Proteomics is able to give us information regarding the proteins after all

posttranslational modifications, and determines the present interior situation of the cells. Current proteomic strategies offer qualitative and quantitative analysis of the protein entirety and allow assessment of protein modifications with high coverage.[4,5] Therefore, the use of proteomics to investigate mechanisms of nonlethal plasma effects on living cells could open up a new dimension of basic understanding of plasma-cell interactions.

II. MATERIAL AND METHODS

A. Plasma Source

For plasma treatment, an atmospheric pressure plasma jet (INP Greifswald, Germany) was used (Fig. 1, left). Briefly, in the center of a quartz capillary (inner diameter 1.6 mm) a pin-type electrode (1 mm diameter) is mounted. Argon (technical, 5.0) as the feed gas flows through the capillary (gas flow up to 10 standard liters per minute). A high-frequency (HF) voltage (1.1 MHz, 2–6 kV) is coupled to the center electrode. The plasma is generated from the top of the center electrode and expands to the surrounding air outside the nozzle. The delivered power of the plasma was measured calorimetrically. The thermal output from the plasma jet is almost independent of the power at about 150 mW.[6] For the experiments presented here, a gas flow of exactly 3.8 standard liters per minute was used, resulting in an apparent plasma jet of about 7 mm length and 1–2 mm width.6 Probe measurements have shown that electrical charges are present in a plasma tip. Axial temperature profiles of the plasma jet were obtained by fiber optic temperature measurement (Luxtron, model 755). A temperature-dependent fluorescent signal of luminescent magnesium fluorogermanate, which was excited with a Xe flashlamp, was monitored (Fig. 2).[7] Optical emission spectroscopy (OES) of the plasma was

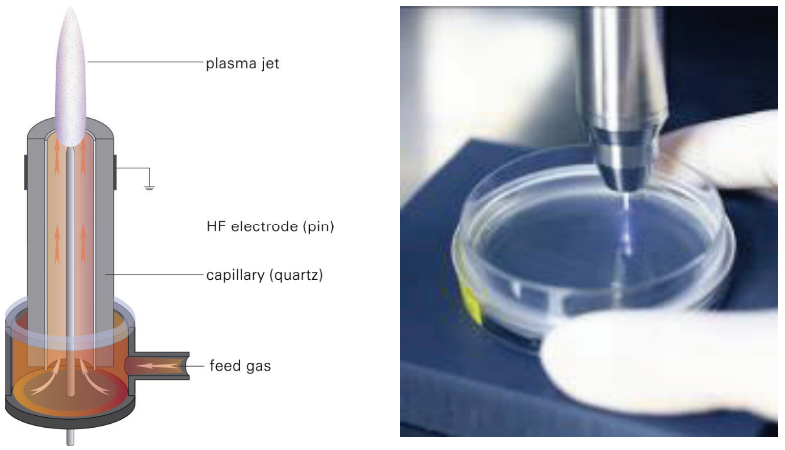

FIGURE 1. Schematic setup of the APPJ plasma source (left), and APPJ treatment of suspended cells in a Petri dish (right).

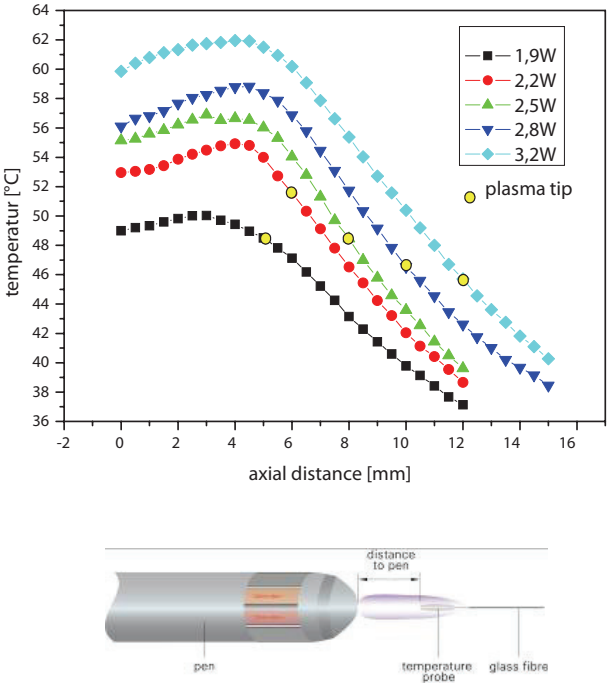

FIGURE 2. Gas temperature of the APPJ (continuous operating mode) and temperature measurement setup at an argon gas flow rate of 5 standard liters per minute dependent on the input power and the axial distance from the capillary nozzle of the plasma source (yellow points: tip of the visible range of the plasma jet).[6]

performed using a fiber spectrometer (StellarNet EPP2000-UVN). The plasma was imaged on the optical fiber of the spectrometer via two pinholes (d = 1 mm) and a quartz glass lens. By moving the optical setup along the plasma, spectra were taken at different but well defined axial positions with a spatial resolution of about 1 mm (Fig. 3).[6] The spectral sensitivity of the detector was taken into account by calibrating it against a deuterium and a halogen lamp.

B. Cell Treatments

Immortalized human keratinocytes (HaCaT) were cultivated in cell culture medium RPMI 1640 (Roswell Park Memorial Institute, Lonza, Verviers, BE) supplemented with 1% penicilline/streptomycine (Biochrom, Berlin, Germany) and 8% fetal calf serum (Sigma-Aldrich, Deisenhofen, Germany) at 37°C and 5% CO_2 in a humidified atmosphere.[8] RPMI is a culture medium for eukaryotic cells. It contains inorganic salts, glucose, amino acids, and vitamins. HaCaT cells were a gift of DKFZ Heidelberg, Germany.

1.8 Mio HaCaT cells in a 75 cm^2 tissue culture flask were grown for four days at 37°C and 5% CO_2 in a humidified atmosphere. On the fourth day, the cells were detached

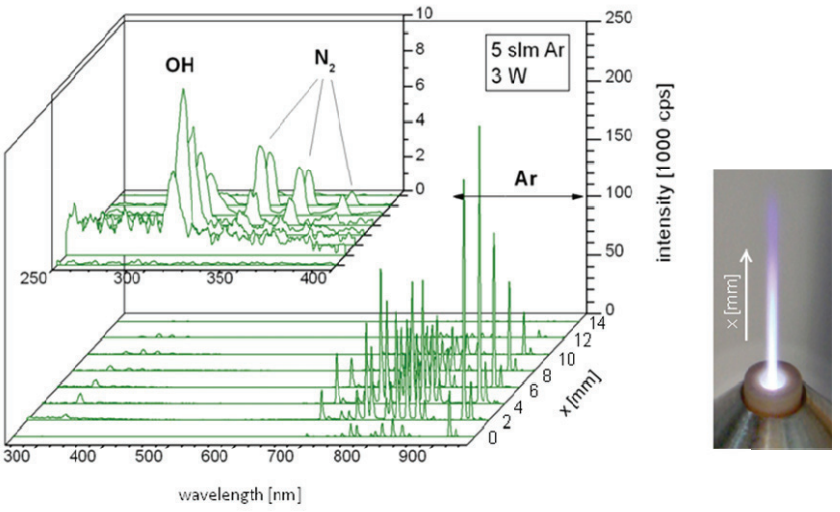

FIGURE 3. Optical emission spectra measured at different axial positions of the Ar-plasma jet.[6]

and suspended in a 5.5 cm diameter Petri dish containing 4 ml RPMI.

Two different experiments were performed related to a control: a 60 s plasma treatment and 60 s gas treatment. The entire Petri dish containing the cell suspension was plasma treated in a meandered track using an automatic program. The speed of the plasma jet over ground was 10 mm/s, resulting in a complete treatment time of 60 s. The plasma jet was adjusted in such a way that the visible plasma tip contacted the liquid surface (Fig. 1, right). The temperature was around 50°C in the visible plasma tip (Fig. 2, green line). For gas flow treatment, the same procedure was used, but without plasma ignition. The gas temperature was about 22°C. All cell treatments started after a 5 min warm-up period. During the treatment, there was no increase in temperature of the culture medium. Lethality of the cells was not observed under these conditions.

C. Proteomics

Immediately after plasma treatment, the cell suspension was centrifuged and the pelletized cells were suspended with urea buffer (8 M urea, 2 M thiourea) and frozen in liquid nitrogen. Protein extraction was performed by a thawing and freezing procedure. Briefly, frozen samples were processed by shaking the tubes in a thermomixer for 10 min at 30°C at 1400 rpm and frozen immediately again in liquid nitrogen. This procedure was carried out five times. Afterward, the cell debris was removed by centrifugation (20,000 × g, 60 min, 4°C). The supernatants were transferred into new tubes, and the protein concentrations were determined with a Bradford assay (Bio-Rad, Munich, Germany).[9] This sample preparation is a standard and well-proved method.[10]

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the composition of two high-resolution electrophoresis procedures to provide much greater resolution than either procedure alone. In the first-dimension gel, solubilized proteins are separated along a pH gradient according to their isoelectric point (pI). The pI is equal to that pH where a given molecule has no net electrical charge. After this first separation, the proteins are separated in the second dimension dependent on their molecular weight. The result of the 2D-PAGE separation is a 2D gel presenting the pattern of the proteome of the cell type investigated.

For the 2D electrophoresis, the samples were rehydrated on IPG strips (24 cm; GE Healthcare, Munich, Germany) with a pH range from 4 to 7. Following a standard protocol previously described by Brigulla et al., the IPG strips were subjected to isoelectric focusing.[11] In the second dimension, proteins were separated on 12.5% SDS-polyacrylamide gels. The resulting gels were stained with colloidal Coomassie brilliant blue according to the manufacturer's instructions (GE Healthcare) and scanned with a light scanner (Epson Perfection V750 Pro).

Using Delta 2D software (Version 3.6, Decodon GmbH, Greifswald, Germany) allows to positionally correct gel images (image warping), spot detection, quatitation, and normalization of spot patterns of numerous gel images by overlay.[12] After defining a master gel, each gel can be compared with the mastergel while the non–master gels are warped automatically using the differences in spot positions between each gel and the master gel. The software calculates and adjusts the same protein spots on gels exactly. Manual corrections may be made. Identical spots are shown black, and spots that appear only in one gel are labeled with different stains.

III. RESULTS AND DISCUSSION

To analyze differences of the proteome of plasma-treated and untreated human keratinocytes (HaCaT cells), images of the 2D gels from the different preparations were overlaid (image warping) and variously colored using the software Delta-2D (Fig. 4: untreated, blue; plasma treated, orange).

Proteins that are not affected by plasma treatment appear black in this projection, which means that they are always in the same position in the different 2D gels. Furthermore, it is possible that constitutively expressed proteins change their position after treatment due to chemical modifications or changes in molecular mass. Plasma-affected proteins appear blue if they are only visible in the untreated control, implying that they are repressed after plasma treatment, or they appear orange if only visible in the plasma treated preparation, implying that they are induced by plasma treatment.

Comparing the proteome images of untreated and plasma treated keratinocytes (Fig. 4), there can be found mainly horizontal shifts of protein spots. These differences of proteins in the first separation dimension indicate changes of the charge state of those proteins resulting in a pI shift. Such changes of the protein charges could be caused by variations of functional groups or ligands by oxidation, methylation, phosphorylation, etc. However, because no mass-dependent vertical shifts of protein spots in the second

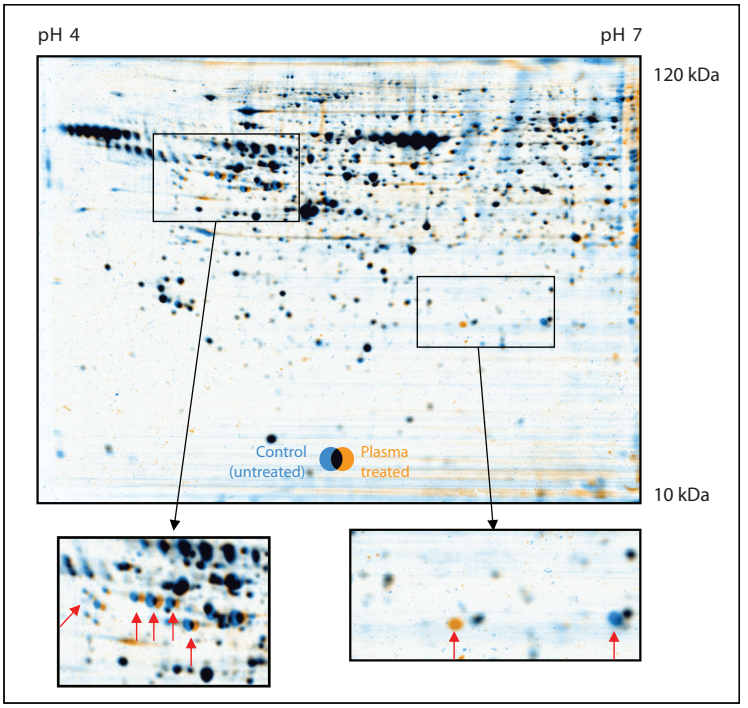

FIGURE 4. Dual-channel overlay image of 2D gels of the protein pattern of untreated (blue) and plasma-treated (orange) keratinocytes. Black spots indicate identical non-modified proteins from both preparations. Blue and orange spots indicate modified proteins being different in the different gels (labeled additionally by red arrows)

dimension of the 2D-PAGE separation are obvious, structural protein changes such as fragmentation, degradation, complexation, etc., caused by plasma treatment, at least under the conditions tested here, can be excluded.

To eliminate effects caused by carrier gas flow, HaCaT cells were exposed to argon gas flow without ignited plasma in an additional experiment. In Figure 5, the same control as in Figure 4 is compared with a cell suspension that was treated only with the gas flow. The spots changed by plasma treatment were unaffected by gas flow, i.e., all spots are black, so it can be concluded that the gas flow itself has no effect on the cells.

As was published elsewhere, radiation emitted from the APPJ plasma contains molecular bands of OH-radical and lines of excited argon atoms between 500 and 1000 nm. In the UV-A region between 350 and 400 nm, bands of nitrogen emission have been measured because of increasing mixing of the feed gas argon with the surrounding ambient air. There was no detectable emission in the UV-C range <250 nm (Fig. 3).[6] Therefore, effects resulting from the well-known intensive biological activity, especially of UV-C around 254 nm, which is based on direct impact on DNA, can be largely precluded.

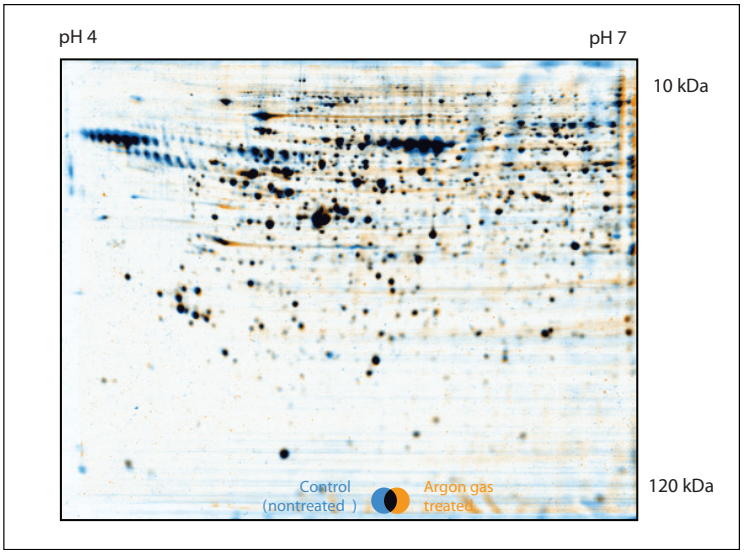

FIGURE 5. Dual-channel overlay image of 2D gels of the protein pattern of untreated (blue) and argon gas treated (orange) keratinocytes. Black spots indicate identical non-modified proteins from both preparations. Blue and orange spots indicate modified proteins being different in the different gels.

Moreover, APPJ emits a significant amount of VUV radiation, mainly the second continuum of the argon excimer Ar_2^* between 120 and 135 nm.[13] The absolute VUV radiance of the APPJ reaches maximum values of 2.2 mW mm^{-2} sr^{-1}.[13] Because the plasma jet is operated in its own argon atmosphere, a considerable amount of VUV radiation can reach an object to be treated in principle. However, because of the fact the cells in the experiment presented here are suspended in a liquid environment, the biological effects of VUV irradiation seem to be unlikely because of its short effective reach and high absorption by air and liquids.[14]

Finally, temperature is one of the most critical factors in connection with plasma treatment of living systems. A slight heating from 37 to 38.5°C was shown to be a stimulus for keratinocyte proliferation.[8] In principle, temperature should not exceed a threshold of 40°C if living systems are treated. In the experimental setup used in this study, the temperature at the tip of the plasma jet was around 50°C (Fig. 2).[6] In principle, this is very close to biological intolerance. However, plasma-caused target heating is not only dependent on gas or plasma temperature, but is also a function of contact time. So, it could be demonstrated that fast-moving atmospheric microwave plasma with a temperature of about 3000°C can be used for decontamination of heat-sensitive hollow packaging materials that are melting above 70°C.[15] Because the cell treatment was realized by moving the APPJ for 60 s along a meandered pattern across the complete surface

of the cell suspension in the Petri dish (area 23.8 cm^2), inducing an additional stirring of the cell suspension, the contact time should be short enough and cooling effects should be sufficient to exclude temperature effects. A substantial warming of the Petri dish containing the cell suspension was not detectable after plasma treatment. However, further experiments should address this problem in a more detailed manner.

IV. CONCLUSION AND OUTLOOK

For the first time, proteomics have been used to investigate the influence of atmospheric pressure plasma on mammalian cells. With it, a very powerful bioanalytical technique has shown its usefulness for basic research in the field of plasma medicine. The pilot experiments presented here indicate the possibility to detect minute changes within vital cells after plasma treatment in a sophisticated manner. Moreover, based on the preparative electrophoretic separation realized by the 2D-PAGE technique, single proteins of interest can be identified by MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry). This was already performed with the gels presented here and is still part of an ongoing investigation.

With the experimental setup used in this study, only effects on cellular proteome immediately after plasma treatment have been demonstrated. To get more detailed insight into possible biochemical mechanisms influencing cell performance, cell reactions have to be investigated up to 72 h after plasma treatment. This will be the only way to get insight into the integral cellular answer and to seize hold on the wobbling expression patterns of proteins and the extent of cellular repair mechanisms in the course of time. Currently, we are preparing this type of experiment, and results will be presented in due time.

One intention of such an examination is the identification of reference proteins to assess a cell status after plasma treatment more easily and to verify the harmlessness of physical plasma to mammalian cells during medical approaches, e.g., in wound healing or dermatology. Hence, proteomics will be a valuable tool to screen effects of plasma treatment on cellular proteome under varying conditions. From its further use in plasma medicine research, detailed knowledge of interactions between physical plasma and cell structures or functions will be obtained. With the first results presented here, the importance to examine internal cellular changes caused by plasma treatment to elucidate the influence of plasma on the metabolism of human cells next to morphological changes, cell performance, and cell viability could be demonstrated.

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REFERENCES

1. Fridman G, Friedman G, Gutsol A, Shekhter AB, Vasilets V, Fridman A. Applied plasma medicine. Plasma Process. Polym. 2008;5:503-533
2. Laroussi M. Low-temperature plasmas for medicine? IEEE Trans. Plasma Sci. 2009; 37:714-725
3. Anderson NL, Anderson NG. Proteome and proteomics: New technologies, new concepts, and new words. Electrophoresis. 1998;19:1853-1861
4. Ferguson PL, Smith RD. Proteome analysis by mass spectrometry. Annu. Rev. Biophys. Biomol. Struct. 2003;32:399-424
5. Amoresano A, Carpentieri A, Giangrande C, Palmese A, Chiappetta G, Marino G, Pucci P. Technical advances in proteomics mass spectrometry: identification of post-translational modifications. Clin. Chem. Lab. Med. 2009;47:647-665
6. Weltmann K-D, Kindel E, Brandenburg R, Meyer Ch, Bussiahn R, Wilke Ch, von Woedtke Th. Atmospheric pressure plasma jet for medical therapy: Plasma parameters and risk estimation. Contrib. Plasma Phys. 2009;49:631-640
7. Foest R, Kindel E, Lange H, Ohl A, Stieber M, Weltmann K-D. Rf capillary jet – a tool for localized surface treatment. Contrib. Plasma Phys. 2007;47:119-128
8. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J. Cell Biol. 1988;106:761-771
9. Deroee AF, Oweinah J, Naraghi M, Hosemann W, Athari B, Völker U, Scharf Ch. Regression of polypoid nasal mucosa after systemic corticosteroid therapy: A proteomics study. Am. J. Rhinol. 2009;23:480-485
10. Hammer E, Bien S, Gesell Salazar M, Steil L, Scharf C, Hildebrandt P, Schroeder HWS, Kroemer HK, Völker U, Ritter CA. Proteomic analysis of doxorubicin-induced changes in the proteome of HepG2cells combining 2-D DIGE and LC-MS/MS approaches. Proteomics. 2010;10:99-114
11. Brigulla M, Thiele T, Scharf Ch, Breitner-Ruddock S, Venz S, Völker U, Greinacher A. Proteomics as a tool for assessment of therapeutics in transfusion medicine: evaluation of prothrombin complex concentrates. Transfusion. 2006;46:377-385
12. Berth M, Moser FM, Kolbe M, Bernhardt J. The state of the art in the analysis of two-dimensional gel electrophoresis images. Appl. Microbiol. Biotechnol. 2007;76:1223-1243
13. Lange H, Foest R, Schäfer J, Weltmann K-D. Vacuum UV radiation of a plasma jet operated with rare gases at atmospheric pressure. IEEE Trans. Plasma Sci. 2009;37:859-865
14. Brandenburg R, Lange H, Woedtke Th, Stieber M, Kindel E, Ehlbeck J, Weltmann K-D. Antimicrobial effects of UV and VUV radiation of nonthermal plasma jets. IEEE Trans. Plasma Sci. 2009;37:877-883
15. Ehlbeck J, Brandenburg R, von Woedtke Th, Krohmann U, Stieber M, Weltmann K-D. PLASMOSE – antimicrobial effects of modular atmospheric plasma sources. GMS Krankenhaushygiene Interdisziplinär. 2008;3:Doc14.