EUKARYOTIC CELLS AS BIOMARKERS FOR COLD PLASMA-GENERATED REACTIVE SPECIES

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ABSTRACT

In the field of Plasma Medicine gas discharges and biology are inevitably linked. However, as new sources and developments evolve there is an increasing need for assays screening for plasma effects on cells. In turn, by assessing certain properties eukaryotic cells can be regarded as biosensors for plasma properties e.g. reactive oxygen species. We here present assays which were designed to help deciphering how plasma influences cells. For this, we used an atmospheric pressure argon plasma jet and human blood cells and investigated cellular viability and oxidation. We show that care must be taken when designing and interpreting plasma experiments involving cells and provide a hypothesis to dismantle the mechanism of plasma mediated cytotoxicity. These may be useful for other researchers who may use different plasma sources and cell types but are in need to characterizing their individual plasma-cell interaction.

1. INTRODUCTION

Due to astonishing scientific progress, numbers and potential applications of plasma sources have increased in recent years. However, many questions regarding plasma-cell interactions have only partially been addressed or successfully answered. At the same time, there is an increasing need in physics to learn about those mechanisms as they are indispensable for tailoring plasma sources and properties for different applications. For example, antibacterial properties of plasma may be more suitable in chronic wound treatment than in cancer cell treatment. We here used human lymphocytes and flow cytometry / plate reader to assess cellular viability and oxidation. Blood lymphocytes display many advantages over adherent cells in terms various investigations as they i) are in suspension which permits a direct plasma treatment also with plasma jets (adherent cells are killed by the gas effluent due to drying effects of the argon flux); ii) are easy to handle and robust e.g. against fluctuating temperature conditions (cell lines require 37°C and cannot withstand lower temperatures for few hours); and iii) display the highest sensitivity to plasma oxidation or toxicity compared to monocytes [1] or ten cell lines (unpublished observation).

In our hands, cellular viability and oxidation are ideal parameters to screen for plasma settings suitable for the given application. Both can be easily investigated on a population basis (plate reader) or single cell level (flow cytometry). In general, flow cytometry allows addressing more complex questions in need of a high sensitivity while plate reader assays are very fast and ideal screening tools. As we have previously investigated human blood T helper cells and monocytes regarding viability and oxidation [2] we here examined oxidation of human cytotoxic T cells after plasma treatment. These cells are crucial in controlling and killing tumor, infected, or otherwise “stressed” cells via recognition of designated cell surface receptors.[3] In this work we asked the following questions: i) what is the relation of plasma cytotoxicity and cell concentration; ii) is there a protective effect of proteins; iii) what is the role of hydrogen peroxide in plasma-cell interaction; and iv) how does this relate to intracellular oxidation after plasma treatment. The methods used and described are applicable to other cell types and hopefully will help researchers to individually characterize their plasma-cell interactions.

2. MATERIALS AND METHODS

Plasma treatment was carried out using an atmospheric pressure argon plasma jet (kinpen 09, neoplas GmbH) and an argon flux of three standard liters per minute as described in detail before.[4, 5] A voltage of 2–6 kV pp was used with a frequency of 1.1 MHz.

Isolation of peripheral blood mononuclear cells (PBMC) was done using donor blood and density gradient centrifugation (lymphocyte separation medium PAA). For treatment, cells were seeded in 24 well plates (Nunc) at 10^6 per ml medium containing 2 % glutamine, 1 % penicillin/ streptomycin, and ± 10 % fetal bovine serum (Biochrom). Upon blood donation at the blood bank (University Medicine of Greifswald, Germany) healthy blood donors gave written and informed consent about anonymous usage of blood for research purposes in the case that the donation is not suitable for clinical application.

Cellular viability and oxidation were measured by flow cytometry (Gallios, Beckman-Coulter) as described before.[2, 4] Briefly, viability was assessed using Annexin V (FITC conjugated, Biolegend) and DAPI 24 h after treatment after staining with anti-CD3 APC (Biolegend) and gating on all CD3-positive lymphocytes. Oxidation was assessed by staining PBMC with CM-H_2DCFDA (Molecular Probes), DAF-FM (Molecular Probes), DHR123 (Sigma) and anti-CD8 (Biolegend) according to vendor's instructions. Fluorescence in CD8-positive cytotoxic T lymphocytes was measured 0-90 min after plasma treatment. In viability and oxidation experiments, catalase (5 µg/ ml, Sigma) was added before plasma exposure to the cell suspension.

In supernatants of PBMC, plasma-produced hydrogen peroxide (H_2O_2) was quantified using Amplex UltraRed reagent (Molecular Probes) and a standard curve of hydrogen peroxide (Sigma) 1 h and 24 h after treatment. Samples were measured in triplicates in black 96 well plates (Nunc) in a plate reader (ex. 530 / em. 590, Tecan).

Data analysis and statistics was done using Kaluza (Beckman-Coulter), excel 2010 (Microsoft), and prism 6.04 (graph pad software).

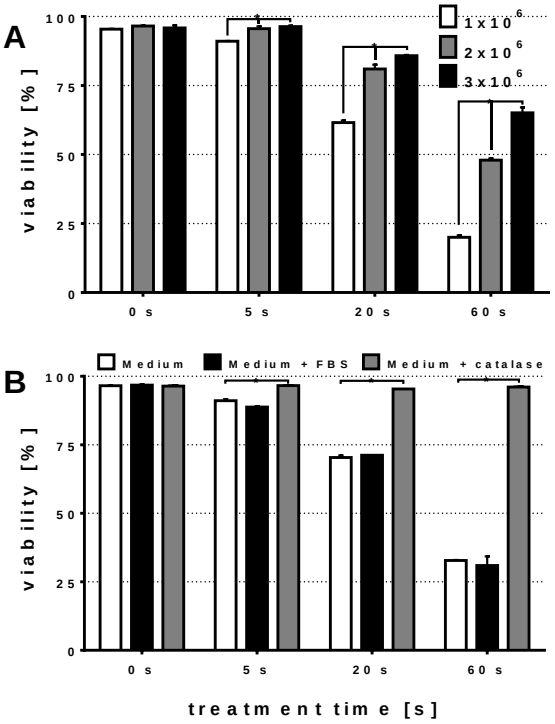

*Fig. 1 Viability of CD3$^+$ T lymphocytes 24 h after plasma treatment using different cell concentrations (A) or altering cell treatment conditions (B) using FCS and catalase. Data are presented mean ± S.D. of duplicates of one representative experiment of three; * denotes $P < 0.05$ was determined by two-way ANOVA.*

3. RESULTS AND DISCUSSION

In this work, we utilized fluorescent indicators of oxidation and viability to characterize plasma properties in a biological system using human lymphocytes. First, we investigated viability of lymphocytes positive for the T cell receptor (CD3) with regard of the cell concentration used. As we have shown before, plasma-cytotoxicity increased in a treatment time dependent manner (Fig. 1A).[4] Interestingly, toxicity decreased with increasing cell concentrations being treated for the same treatment time. Cells possess an anti-oxidative potential and readily react with plasma components as reactive oxygen species (ROS). By applying different cell concentrations investigators can estimate this potential. For cytotoxic T cells, this effect was significant.

To assess cytotoxic protection of proteins in cell culture medium via reaction and thus detoxification of ROS we added 10 % FBS and compared results (Fig. 1B). As viability was similar, we did not find a guarding role of non-

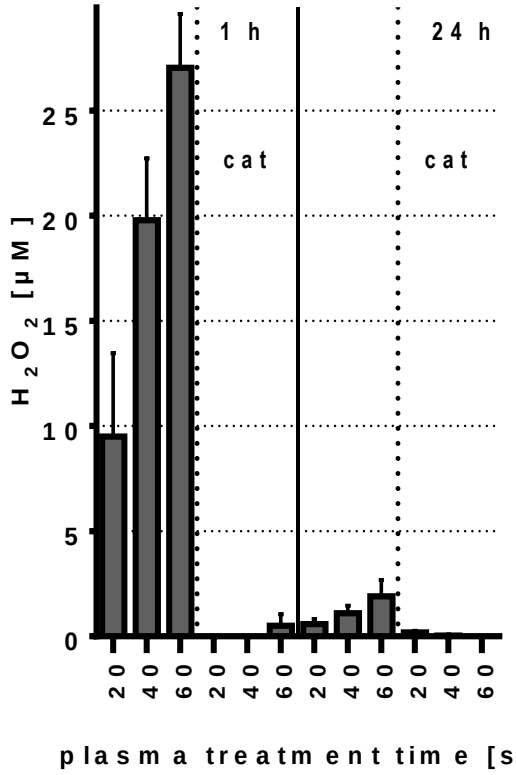

Fig. 2 Quantification of H_2O_2 in supernatants of plasma-treated PBMC. H_2O_2 was clearly present 1 h after plasma treatment but only minor concentrations were detectable 24 h after treatment. Catalase (cat) fully scavenged H_2O_2. Data are presented mean ± S.E. of 6 independent experiments.

radical-specific proteins in our plasma-treatment system. On the other, the H2O2-degrading enzyme catalase fully and significantly protected plasma-treated $CD8^+$ cells from going into apoptosis. We have previously shown similar effects in human T helper cells.[2] As catalase degrades hydrogen peroxide this stable oxidant is very likely the main mediator of plasma cytotoxicity and it has been found in many plasma sources before.[6-8] We next sought to quantify H_2O_2 in supernatants of plasma-treated PBMC (Fig. 2). We found 27 µM H_2O_2 1 h after exposure (1 min treatment). As we previously have measured the H_2O_2 production rate in our treatment system (1 min ~ 60 µM)[2] 33 µM H_2O_2 have decayed or were scavenged by cells within one hour. This number may be different for other cell types and conditions used. Only residual concentrations were detectable 24 h after treatment. Therefore, it can be speculated that supernatants of only-just plasma treated cells will be toxic to other cells as while after 24 h they will not. Finally, catalase fully inhibited high H_2O_2 concentrations in plasma exposed PBMC suspension and also confirmed that fluorescence increase of oxidation dye was due to H_2O_2.

After investigating the viability of T lymphocytes we then measured the intracellular change in redox state via three different probes (Fig. 3). In addition to plasma treatment we used a concentration-matched control of H_2O_2 (60 µM) to investigated how much of the plasma-induced oxidation can be addressed to H_2O_2 alone. All probes reacted with plasma and experimentally added H_2O_2. Catalase diminished most but not all of fluorescence increase in all probes for plasma treated samples. It is possible that the residual fluorescence increase is due to other species being generated by plasma. This principle is of note and could also serve as an indirect proof of oxidizing species other than H_2O_2 of and in maybe more potent plasma sources. While DCF and DHR123 serve as general and therefore non-specific oxidative stress indicators, DAF-FM is attributed specificity towards nitrogen monoxide. We have seen similar non-specificity for so-called specific probes before.[2] Therefore, care must be taken when interpreting results of fluorescent redox marker and proper controls for H_2O_2 should always be included as it is likely that it will be present in plasma treated samples in high concentrations. Although 60 s of plasma treatment leads to the deposition of 60 µM of H_2O_2 [2] plasma treated samples always fluoresced similarly or higher than H_2O_2 treated samples which is an additional indication of oxidation of species other than H_2O_2. For DCF this could be hydroxyl radical or peroxynitrite,[9] both short living species directly or indirectly proven to be produced or induced by kinpen 09 operation.[2, 6]

4. SUMMARY

Intracellular oxidation and cytotoxicity are suitable parameter to characterize plasma-cell interaction for any given source or cell type. Both can be quantified using fluorescent markers and are thus detectable with a plate reader or by flow cytometry. By introducing proper controls e.g. specific oxidant scavenger as catalase, contribution of various plasma-produced oxidants on cells can be investigated. We clearly demonstrated a dominant effect of H_2O_2 in plasma-induced oxidation and cytotoxicity in human blood cells for the kinpen 09. This, however, may be different for other plasma sources used and could be investigated using the approach described.

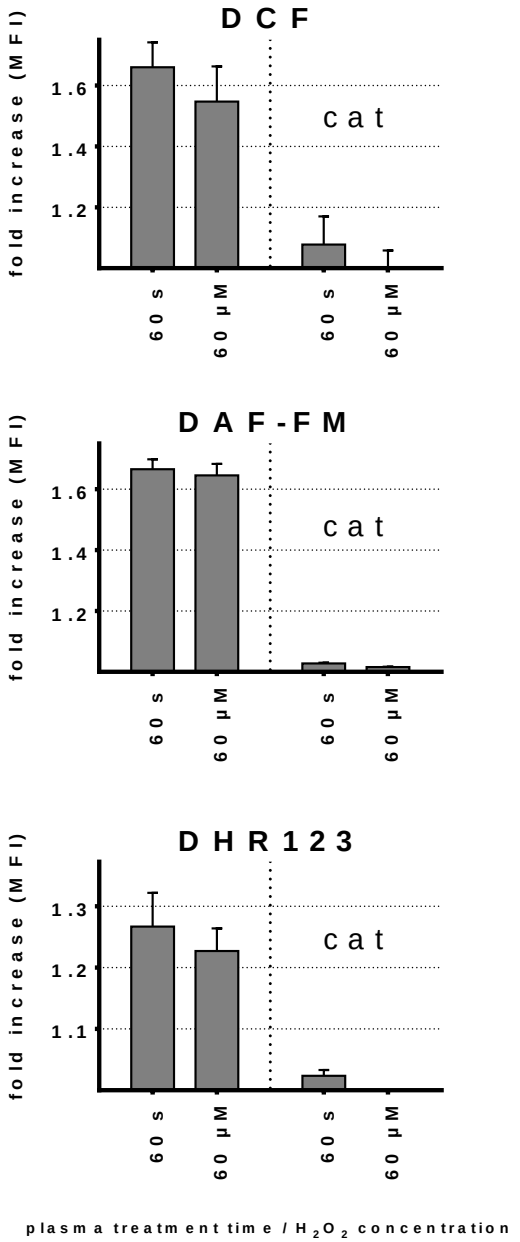

Fig. 3 Intracellular fluorescence increase (over control) of three different redox probes in $CD8^+$ cytotoxic T cells. Cell were either treated with plasma (60 s) or H_2O_2 (60 µM). Catalase was added as control before treatment to scavenge H_2O_2. Data are presented mean ± S.D. of duplicates of one representative of three to four experiments.

5. ACKNOWLEDGMENTS

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