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Original Article

Cold atmospheric plasma, a new strategy to induce senescence in melanoma cells

Stephanie Arndt[1], Eva Wacker[1], Yang-Fang Li[2], Tetsuji Shimizu[2], Hubertus M. Thomas[2], Gregor E. Morfill[2], Sigrid Karrer[3], Julia L. Zimmermann[2] and Anja-Katrin Bosserhoff[1]

[1]Institute of Pathology, University of Regensburg, Regensburg, Germany; [2]Max Planck Institute for Extraterrestrial Physics, Garching, Germany; [3]Department of Dermatology, University of Regensburg, Regensburg, Germany

Correspondence: Anja-Katrin Bosserhoff, PhD, Institute of Pathology, University of Regensburg, Franz-Josef-Strauss-Allee 11, D-93053 Regensburg, Germany, Tel.: (+49)-941-944-6705, Fax: (+49)-941-944-6602, e-mail: anja.bosserhoff@klinik.uni-regensburg.de

Abstract: Over the past few years, the application of cold atmospheric plasma (CAP) in medicine has developed into an innovative field of research of rapidly growing importance. One promising new medical application of CAP is cancer treatment. Different studies revealed that CAP may potentially affect the cell cycle and cause cell apoptosis or necrosis in tumor cells dependent on the CAP device and doses. In this study, we used a novel hand-held and battery-operated CAP device utilizing the Surface Micro Discharge (SMD) technology for plasma production in air and consequently analysed dose-dependent CAP treatment effects on melanoma cells. After 2 min of CAP treatment, we observed irreversible cell inactivation. Phospho-H2AX immunofluorescence staining and Flow cytometric analysis demonstrated that 2 min of CAP treatment induces DNA damage, promotes induction of Sub-G1 phase and strongly increases apoptosis. Further, protein array technology revealed induction of pro-apoptotic events like p53 and Rad17 phosphorylation of Cytochrome c release and activation of Caspase-3. Interestingly, using lower CAP doses with 1 min of treatment, almost no apoptosis was observed but long-term inhibition of proliferation. H3K9 immunofluorescence, SA-ß-Gal staining and p21 expression revealed that especially these low CAP doses induce senescence in melanoma cells. In summary, we observed differences in induction of apoptosis or senescence of tumor cells in respond to different CAP doses using a new CAP device. The mechanism of senescence with regard to plasma therapy was so far not described previously and is of great importance for therapeutic application of CAP.

Abbreviations: CAP, cold atmospheric plasma; H2AX, Histone H2A.x; H3K9, Histone H3 lysine 9; PCNA, proliferating cell nuclear antigen; DSBs, double-strand breaks; SAHF, senescence-associated heterochromatin foci; FACS, fluorescence-activated cell sorting; NHEM, normal human epidermal melanocytes.

Key words: apoptosis – melanoma cells – plasma – senescence

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Introduction

Cancer is a disease of unlimited cell growth. The ability of a normal cell to self-regulate is a vital function in higher organisms allowing for appropriate, controlled growth. Growth suppression can be achieved by induction of programmed cell death (apoptosis) or by suppressing cell proliferation in other way but without inflicting accidental cell death (necrosis). Apoptosis is executed in old, damaged or not functional cells. It involves a series of biochemical events leading to changes in cell morphology and behaviour, such as chromatin condensation or DNA fragmentation (1). The inactivation of a tumor-suppressor gene that facilitates apoptosis or the over-expression of an anti-apoptotic protein are both important mechanisms promoting cancer development. To treat cancer, many conventional anti-cancer agents are available. DNA-damaging agents, which have been clinically employed for many decades, target DNA and cause irreparable DNA double-strand breaks (DSBs). Eventually, these DSBs cause cell cycle arrest, apoptosis and rapidly increasing cancer-specific cell death. Within 30 min after DSB formation, the core histone protein variant H2AX is phosphorylated and localizes DNA-damaged sites (2,3). The gamma-H2AX signal is therefore a specific marker indicating DNA damage.

Melanoma is one of the malignancies with highest therapy resistance, and regardless of new therapeutic attempts, the outcome remains dismal. With an incidence that is increasing at 2–5% per year, cutaneous melanoma disproportionately targets young individuals. Despite a lot of research, the treatment of this advanced disease is still quite challenging.

Recently, the effects of cold atmospheric plasma (CAP) have been proposed as a novel therapeutic method for anti-cancer treatment; however, its biological effects and mechanisms of action remain elusive (4,5). There a several studies which describe that suitable CAP doses usually do not induce necrosis, but trigger sub-lethal or lethal cell reactions (detachment, apoptosis) in various cell types (6–8). Other studies describe necrosis or degradation of adhesion molecules of tumor cells after the CAP treatment (9,10). In general, it is difficult to compare the results of different CAP studies, due to the application of different CAP devices and plasma parameters and therefore different compositions and concentrations of the plasma products – electrons, charged particles, reactive species, UV light and heat. The conceptual possibility to design CAP and treatment parameters to ensure its effectiveness and selectivity regarding its action on different types of living organisms and cells has become a subject of interest and active research during the last several years.

The purpose of this study was to evaluate the influence of CAP using a newly developed plasma device based on the Surface Micro Discharge (SMD) technology (11,12) on malignant

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melanoma. Our findings provide new ideas for the cellular mechanisms induced by CAP in melanoma cells and suggest an alternative therapeutic approach to melanoma.

Materials and methods

Plasma device and treatment of cells

The CAP device employed in this study (short name 'miniFlatPlaSter') uses the flexible and scalable Surface Micro Discharge (SMD) technology for plasma production in air (11) and has been published in Maisch et al. (12). The cells were treated with CAP in 6-well plates, 16-well E-plates or 8-well chamber slides without cell culture medium covering the cells. During the treatment, the CAP device – possessing an electrode of 28 mm in diameter – was placed directly onto the plates to generate a closed system to confine the created species (mainly reactive oxygen and nitrogen species) inside. Control cells remained untreated. Medium was added to all wells immediately after CAP exposure. The main components produced by the CAP device are listed in Table S1. The reactive species produced by the CAP device are transported to the cells via diffusion. Due to this treatment method, drying effects of the cells can be explicitly ruled out – in contrast to cell culture experiments where plasma jets, torches, etc. are applied – and even cells only covered with a thin film of medium can be treated and analysed. The used CAP source – in contrast to Dielectric Barrier Discharge (DBD) devices – produces very low currents (<50 nA/cm^2), which if applied *in vivo* ensures a safe application.

Cell culture

The melanoma cell lines used for this study have been described previously (13). The cell lines Mel Juso, Mel Ei and Mel Ho were derived from a primary cutaneous melanoma, whereas Mel Im, Mel Ju and HTZ19 were obtained from metastases of malignant melanoma. All cell lines were cultured according to 13.

Normal human epidermal melanocytes (NHEMs) (PromoCell, Heidelberg, Germany) were derived from neonatal skin and cultivation was described previously (14). Cells were used between passages 2 and 4 and not later than 2 days after trypsinization.

xCELLigence system

The xCELLigence System (Roche, Mannheim, Germany) is an innovative method based on the measurement of electrical impedance to analyse cells in real time as previously described (15). The electrical impedance represents the cell status (cell viability, cell proliferation). Impedance was represented by the relative and dimensionless parameter named cell index (CI). Further information on technology is given on the manufacturer's homepage (www.xcelligence.roche.com).

Melanoma cells (Mel Im and Mel Juso) were seeded at a density of 3000 cells per well as duplicates into a 16-well E-Plate, covered with 100 μl DMEM and placed into the Real-Time Cell Analyzer (RTCA) station. The adhesion, spreading and proliferation of the cells were dynamically monitored for approximately 24 h by measuring the impedance continuously. Afterwards, the cell culture medium was removed from all wells, and the upper 6-wells were treated with CAP for 1 or 2 min in a closed system. The 4 median wells were cell-free empty controls and were used to determine the baseline cell index. The lower 6-wells remained untreated. Immediately after the CAP treatment, medium was added to each well, and the cell behaviour was dynamically monitored every 10 s, 1 h long and afterwards every 10 min for up to 90 h. All experiments were performed in duplicates and were repeated 3 times.

Cell cycle analysis

For cell cycle analysis and Sub-G1 determination analysis by flow cytometry, 20 000 cells were seeded into 6-well plates and treated with CAP for 1 or 2 min in a closed system. After 20 h, cells were fixed in ice-cold 70% ethanol for at least 30 min, washed twice with phosphate/citrate buffer (192 mM Na_2HPO_4, 40 mM citric acid), treated with RNase A, stained with propidium iodide and analysed using a FACSCanto II cytometer (Becton Dickinson, Heidelberg, Germany).

Apoptosis analysis

For analysing apoptosis, cells were cultured and treated with CAP in analogy to the cell cycle analysis. Apoptotic cells were investigated by flow cytometry using the ApoDETECT ANNEXIN V-FITC KIT (Zymed, Carlsbad, CA, USA) according to the manufacturer's instructions. The flow cytometry analysis was performed in a FACSCanto II cytometer (Becton Dickinson, Heidelberg, Germany). FACS data were analysed using FACSDiva software (Becton Dickinson).

For determination of the relative levels of apoptosis-related proteins, the Human Apoptosis Array Kit (ARY009; R&D Systems, Minneapolis, USA) was used. Briefly, Mel Im cells were treated 2 min with CAP, control cells retained untreated. 400 μg of cellular extract from CAP-treated cells and from untreated control cells, both isolated 6 h after exposure, was incubated overnight with the Human Apoptosis Array. All following steps were performed according to the manufacturer's instructions. Detection of the spots was performed on an Image Quant LAS 4000 mini Luminescent Image Analyzer (GE Healthcare) and pixel density of the spots was calculated with the Image Quant TL software (GE Healthcare, Freiburg, Germany).

Senescence-associated ß-galactosidase staining

To determine senescent melanoma cells or NHEMs after the CAP treatment for 1 min, cells were incubated for further 48 h, stained and counted as described previously (16). Representative pictures were collected by light microscopy (Zeiss, Axiovision) in magnifications as indicated.

Immunofluorescence staining

Ten thousand cells/well were seeded into 8-well chamber slides and treated with CAP for 1 and 2 min. 20 h after the treatment, cells were washed, fixed, permeabilized and blocked as described previously (17). Subsequently, cells were incubated with anti-H2AX (phospho-) antibody (1:200; Life Technologies GmbH, Darmstadt, Germany), anti-H3K9 (trimethyl-) antibody (1:200; Merck Millipore, Darmstadt, Germany) or anti-PCNA antibody (1:200; DAKO, Hamburg, Germany) overnight at 4°C. After washing, cells on coverslips were incubated with the secondary antibody (1:40, fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin or FITC-conjugated anti-rabbit immunoglobulin; both from DAKO) for 1 h, followed by rinsing with PBS and co-staining with 80 nmol/L rhodamine phalloidin (F-actin) (Cytoskeleton, Denver, CO, USA) in PBS-containing 10% goat serum for 40 min. After washing with PBS, cells were mounted with Vectashield Hard Set Mounting Medium with DAPI H-1500 (Vector Laboratories, Burlingame, CA, USA). Images were collected by immunofluorescence microscopy using an Axio Imager Zeiss Z1 fluorescence microscope (Axiovision Rel. 4.6.3, Carl Zeiss AG, Oberkochen, Germany, Freiburg, Germany).

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RNA isolation and reverse transcription

Total cellular RNA was isolated from Mel Im and Mel Juso 20 h after the CAP treatment using the RNeasy kit (QIAGEN, Hilden, Germany), and cDNAs were generated by reverse transcriptase reaction as described elsewhere (13).

Analysis of mRNA expression by quantitative RT-PCR

Quantitative real-time PCR for p21 and β-actin was performed by means of LightCycler® 480 II technology (Roche, Mannheim, Germany) according to (18) using following forward and reverse primers from Sigma-Aldrich (p21 fw 5′-CGA GGC ACC GAG GCA CTC AGA GG-3′, p21 rev 5′-CCT GCC TCC TCC CAA CTC ATC CC-3′; ß-actin fw 5′-TGA CGG GGT CAC CCA CAC -3′, ß-actin rev 5′-CTA GAA GCA TTT GCG GTG GAA -3′). β-actin was amplified to ensure cDNA integrity and to normalize expression. Each real-time PCR was performed in duplicates and the whole experiment was repeated 3 times.

Protein analysis by western blotting

Protein analysis was performed as described elsewhere (18) using 60-μg lysate from CAP-treated and from untreated control cells isolated 80 h after exposure for 1 min. Primary antibodies used are: p21 (BD Pharmingen; 1 μg/ml in 5% MP/TBST) and ß-actin (Sigma-Aldrich; 1:5000).

Statistical analysis

Results are expressed as the mean ± SD (range) or a percentage value. Comparisons between groups were made using Student's unpaired t-test. Statistical significance of P-values was represented. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$. All calculations were performed using the GraphPad Prism software package (GraphPad Software Inc., San Diego, CA, USA).

Results

The aim of this study was to evaluate the influence of CAP using a newly developed plasma device based on the Surface Micro Discharge (SMD) technology on malignant melanoma to determine the resulting effects and to understand their molecular basis.

Irreversible melanoma cell inactivation after 2 min of CAP treatment

To determine the effects of CAP treatment on cellular behaviour in real-time, the xCELLigence System (Roche, RTCA) was used. With this technique, real-time changes in electrical impedance across microelectrodes integrated into the bottom of tissue culture E-Plates were analysed up to 90 h after the CAP treatment. As shown in Fig. 1a for Mel Im and Fig. 1c for Mel Juso, 2 min of CAP treatment of exponentially growing melanoma cells causes the cell index to drop dramatically and to remain in a steady state. The control cells without the CAP treatment continue to proliferate in a normal manner. The duration of the CAP treatment is highlighted within a red box. Important to note is that during the treatment, a general influence on the impedance on all wells is seen.

The decrease in the cell index is exemplarily shown 4 h and 20 h after 2 min of CAP exposure of Mel Im (Fig. 1b) and Mel Juso (Fig. 1d). Two minutes of treatment lead to an immediate and long-time irreversible melanoma cell inactivation.

Proliferation arrest was supported by a strong reduction in proliferating cell nuclear antigen (PCNA) immunofluorescence staining analysed 20 h after the CAP exposure for 2 min (Fig. 1e).

Analysing the cell morphology after the CAP treatment for 2 min using light microscopy, a more rounded cellular shape

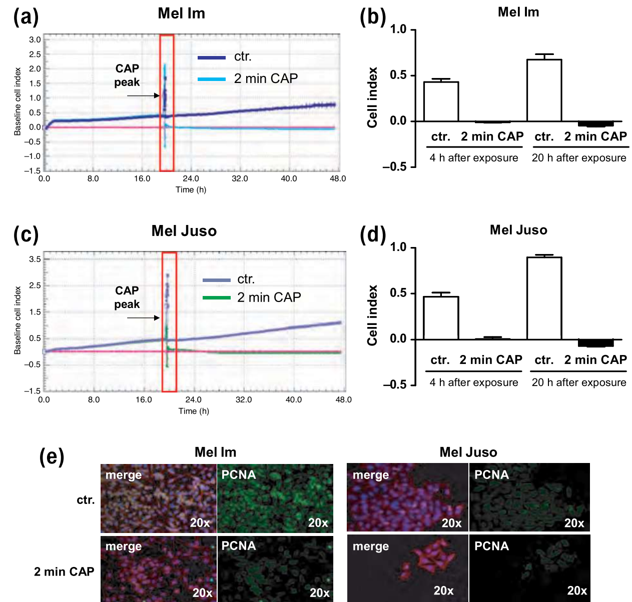

Figure 1. Cell behaviour after 2 min of cold atmospheric plasma (CAP) treatment. Differences in the cell activity between control (ctr.) and 2 min of CAP-treated cells were analysed in the xCELLigence System. Cell viability of 3000 Mel Im (a) and Mel Juso cells (c) per chamber of an E-plate was monitored with the impedance measurement of the xCELLigence System and presented as Baseline Cell Index. Reduction in the Cell Index of Mel Im (b) and Mel Juso (d) after 2 min of CAP treatment was shown exemplarily 4 h and 20 h after exposure compared with the untreated control (ctr.). (e) Representative examples of proliferating cell nuclear antigen (PCNA) (green) immunofluorescence staining of Mel Im and Mel Juso cells 20 h after treatment with CAP for 2 min. The overlap between PCNA (green), DAPI (blue) and F-actin (red) staining is added (merge). Reduction in PCNA (proliferation marker) staining suggests reduction in cell proliferation after CAP treatment.

immediately and up to 4 h after the treatment was observed. However, the cells were viable and did not completely lose their contact to the plate (Figure S1 (a)). These results suggest that necrosis is not induced after 2 min of CAP treatment. However, 20 h after the 2 min of plasma application, most of the cells were reattached, but a significant decrease in living cells in comparison with the control (ctr.) cells without CAP treatment was observed (Figure S1 (b)). These results suggest that apoptosis is the mechanism for cell death.

DNA fragmentation, Sub-G1 accumulation and induction of apoptosis after 2 min of CAP treatment

Immunofluorescence-stained gamma H2AX foci were analysed in Mel Im cells after the CAP treatment of 2 min and further incubation for 20 h compared with the 100-μM etoposide treatment for 3 h (Fig. 2a). Etoposide is a cancer chemotherapeutic drug that induces DNA double-strand breaks (DSBs). The level of gamma-H2AX staining after the CAP treatment was similar to etoposide treatment, suggesting a high plasma-induced DSB formation in Mel Im cells. The data were confirmed by propidium iodide (PI) FACS analysis. A significant proportion of CAP-treated Mel Im and Mel Juso cells showed DNA fragmentation, which was represented by the sub-diploid fraction (Sub-G1) of the cell cycle profile. Approximately 50% of the cells from both cell lines were detected in Sub-G1 20 h after the CAP treatment for 2 min compared with the control (Fig. 2b).

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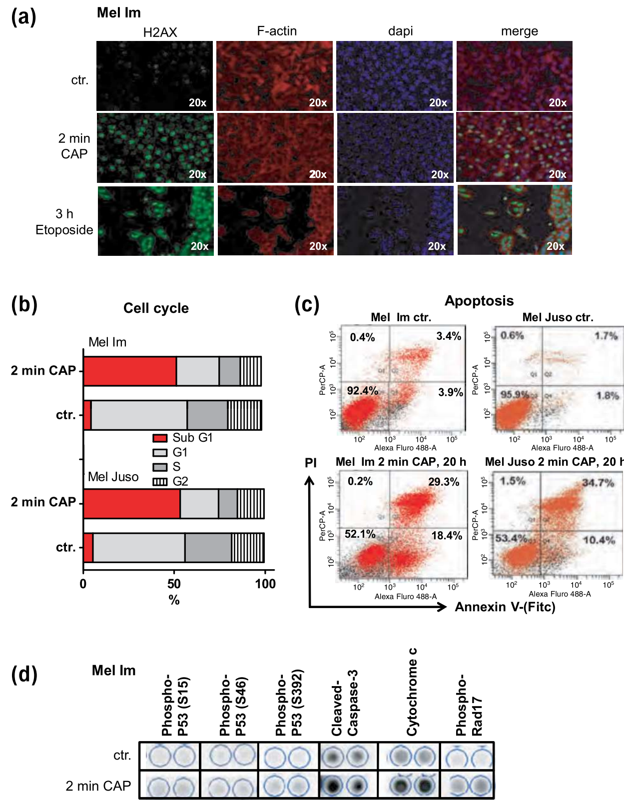

Figure 2. Cellular mechanism after 2 min of cold atmospheric plasma (CAP) treatment. (a) Phosphorylated H2AX (green) immunofluorescence staining following 2 min of CAP exposure and 3 h of treatment with 100 μM etoposide indicate high amounts of double-strand breaks (DSBs) in Mel Im cells. F-actin microfilaments (red) are not modified by the CAP treatment. DAPI (blue) was used for nuclear staining. Merge photographs (H2AX, F-actin, DAPI) are added. (b) Cell Cycle of Mel Im and Mel Juso cells 20 h after 2 min of CAP treatment was examined by flow cytometry. PI staining and cell cycle analysis were performed, and the Sub-G1 fraction was used to quantify apoptotic cells. (c) Annexin V/PI double-staining assay was performed 20 h after 2 min of CAP exposure. The y-axis shows the PI-labelled population, and the x-axis shows FITC-labelled annexin V positive cells. Necrotic cells are Annexin V negative and PI positive (top-left sector), apoptotic dead cells are both PI and Annexin V positive (top-right sector) and apoptotic cells are Annexin V positive and PI negative (lower-right sector), respectively. A typical result from 3 independent experiments is shown. The numbers in the graphs represent the percentage of the cells in the region among the total cells. (d) The Human Apoptosis Array detects multiple apoptosis-related proteins and was performed with 400 μg of Mel Im protein lysate obtained 6 h after 2 min of CAP-treated and untreated control cells. Images from the protein spots in duplicates were collected from 2 min exposures and show an induction of p53 phosphorylation on S15, S46 and S392, Rad17 phosphorylation on S635, Cytochrome c activation and induction of cleaved-Caspase-3.

Induction of apoptosis of different melanoma cells from primary tumors, from melanoma metastasis and from normal melanocytes (NHEMs) after the CAP treatment of 2 min was further confirmed by Annexin V/PI staining and FACS analysis 20 h after the treatment and was exemplarily shown for Mel Im and Mel Juso cells (Fig. 2c). Twenty hours after the CAP treatment, 45.1% of Mel Juso, 27.3% of Mel Ei and 19.8% of Mel Ho cells derived from primary melanomas and 47.7% of Mel Im, 30.2% of Mel Ju and 14.2% of HTZ19 cells obtained from melanoma metastasis underwent apoptosis. These findings suggest that there is no correlation between the extent of malignancy and the effect of the CAP treatment. Furthermore, we observed that normal melanocytes are less sensitive to CAP treatment. Only 9.1% of the cells underwent apoptosis after CAP treatment (data not shown).

To confirm induction of apoptosis-related molecules, an apoptosis protein array was performed with Mel Im protein lysate which was isolated 6 h after the CAP treatment of 2 min. Here, induction of p53 phosphorylation (S15, S46, S392) as well as Rad17 phosphorylation (S635) was observed. In addition, active Caspase-3 and free Cytochrome c were enhanced after the treatment compared with the untreated controls (Fig. 2d). Quantification of the Array results is shown in Figure S2.

Cell inactivation but rarely apoptosis induction after 1 min of CAP treatment

Interestingly, using xCELLigence analysis, we observed that the CAP treatment for only 1 min also led to complete inactivation of Mel Im cells, and almost complete inactivation of Mel Juso cells (Figure S3 (a) and (c)). The cell index 4 h and 20 h after the exposure for both cell lines is presented in Figure S3 (b) and (d). The observed differences between the two cell lines seem to be due to their different sensitivity to the CAP treatment.

By analysing the H2AX foci with immunofluorescence staining, we observed only a marginal number of cells with DSBs 20 h after a CAP treatment of 1 min compared with etoposide-treated cells (Fig. 3a) suggesting that DNA is not damaged by a moderate CAP application rate. F-actin co-staining was added to present the cytoskeleton structure of the cells, but no optical differences were observed between the CAP-treated cells and the control.

Cell cycle (Fig. 3b) and apoptosis (Fig. 3c) analysis was performed 20 h after the 1 min CAP exposure and showed no evidence for induction of apoptosis. Due to these findings, the

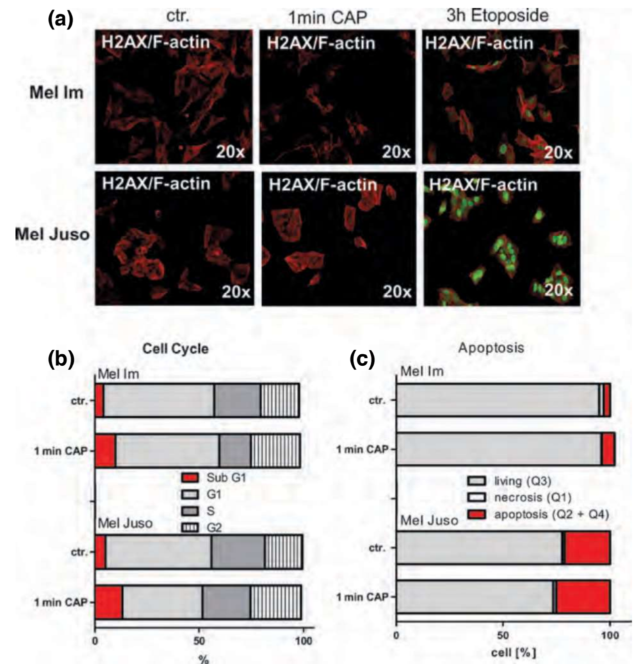

Figure 3. Cell behaviour after 1 min of cold atmospheric plasma (CAP) treatment. (a) Immunofluorescence images of phosphorylated H2AX (green) and F-actin (red) in CAP-exposed Mel Im (upper panels) and Mel Juso (lower panels) in comparison with untreated (ctr.) and etoposide-treated cells for 3 h. Only little phosphorylation of H2AX can be detected, revealing low frequency of DSBs after 1 min of CAP treatment. (b) Cell Cycle analysis of Mel Im and Mel Juso analysed 20 h after 1 min of CAP treatment gives no hint to apoptosis. (c) Flow cytometric analysis of Annexin V-FITC and PI staining revealed no manifest induction of apoptosis in Mel Im and Mel Juso cells 20 h after 1 min of CAP treatment.

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question arose which mechanism leads to the observed sustained cell inactivation without inducing apoptosis.

Induction of senescence is the cause for inactivation of tumor cells after 1 min of CAP exposure

Cellular senescence, by which normal cells lose the ability to divide after about 50 cell divisions *in vitro*, is lost in tumor cells. Tumor cells are usually immortal and senescence is omitted.

To investigate whether the inactivation of tumor cells seen after 1 min of CAP treatment is due to an induction of senescence, we performed H3K9 immunofluorescence staining and senescence-associated ß-galactosidase staining. Interestingly, both staining were strongly induced in Mel Im and Mel Juso cells after 1 min of CAP treatment indicating induction of senescence (Fig. 4a,b).

In analogy to our apoptosis results, we observed no correlation in the number of senescent cells and the progression of malignancy (Figure S4 (a)).

A strong reduction in the cell proliferation after 1 min of CAP exposure was supported by a reduction in proliferating cell nuclear antigen (PCNA) immunofluorescence staining analysed 20 h after the CAP treatment (Figure S4 (b)). Specialized domains of facultative heterochromatin, called senescence-associated heterochromatin foci (SAHF), accumulate in irreversibly proliferation-arrested senescent human cells (19). The existence of irreversibly proliferation-arrested senescent melanoma cells was affirmed by a strong accumulation of SAHF observed in CAP-treated melanoma cells for 1 min compared with the untreated control (Figure S4 (c)).

Furthermore, p21 mRNA (Fig. 4c) and protein (Fig. 4d) analysis indicate p21 activation in Mel Im and Mel Juso cells after 1 min of CAP treatment. p21 has been identified as a senescent cell-derived inhibitor of DNA synthesis (20). Finally, our observations suggest that p21 is also required for senescence development of melanoma cells following the treatment with lower doses of CAP.

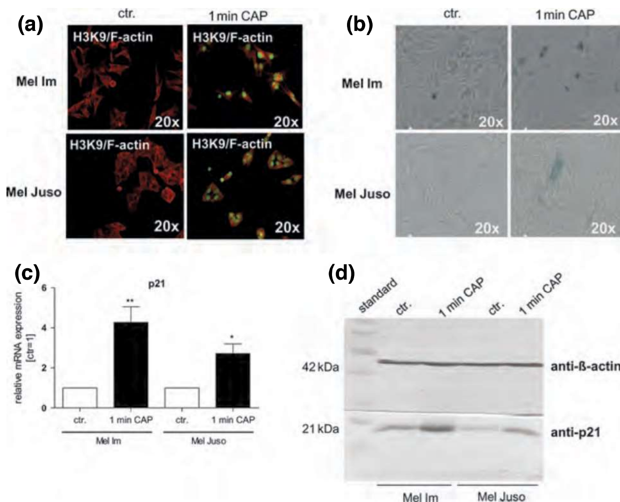

Figure 4. Cellular mechanism after 1 min of cold atmospheric plasma (CAP) treatment. (a) Merged images of Mel Im (upper panels) and Mel Juso (lower panels) stained with antibodies against triMe H3K9 (histone H3 with trimethylated lysine 9) (green) and F-actin (red) 20 h after 1 min of CAP treatment and the untreated control (ctr.). (b) Light microscopic examination of senescence-associated *β*-galactosidase in Mel Im and Mel Juso after treatment with CAP for 1 min and further cultivation for 48 h versus the untreated control (ctr.). Assessment of p21 expression in Mel Im and Mel Juso cells after 1 min of CAP treatment was analysed on mRNA level (c) 20 h after CAP treatment and on protein level (d) 80 h after exposure compared with control (ctr.). $^*P \leq 0.05$, $^{**}P \leq 0.01$.

Discussion

Cold atmospheric plasma (CAP) is an emerging research topic of rapidly growing importance in health care. One promising new medical application of CAP is cancer treatment. Depending on the source (and therefore the used CAP parameters power input, voltage, frequency, carrier gas, etc.), the 'design' and the dosage, there are hints that CAPs act selectively and have different effects on tumor and normal cells.

In the last years, several studies on tumor cells have been published using different kinds of CAP devices, different cell treatment times and experimental setups but most of the studies observed similar effects on tumor cell behaviour. Fridman and colleagues used a floating electrode dielectric barrier discharge plasma in air and described adverse (apoptotic) effects on melanoma skin cancer cells, without damaging the healthy tissue (21). Ahn and co-authors analysed biological effects and application of plasma jets from a micro nozzle array on tumor cells and observed induction of apoptosis via generation of ROS (22). Kim and colleagues observed dose-dependent induction of apoptosis in B16 murine melanoma cells using a 15-μm-sized single cellular level and cell-manipulatable microplasma jet (23), and Lee appropriated a low-temperature radio-frequency atmospheric plasma device, which operates with helium and suggested that CAP reduces melanoma cell adhesion by degradation of adhesion molecules important for malignant transformation and acquisition of metastatic phenotype (10). In summary, independent of the used plasma device, a predominance of apoptotic or necrotic processes are described in relation to CAP contents and composition, and cell treatment times.

We are the first using a large area CAP dispenser based on the so-called Surface Micro Discharge (SMD) technology to analyse dose-dependent effects on melanoma cells. The advantage of the SMD technology is that it is scalable – small, even hand-held and battery-operated devices, but also very large area electrodes can be built easily – depending on the desired application. Furthermore, the plasma is produced indirectly (just using the surrounding air and electricity) and transported to the sample to treat (in this study different tumor cells) via diffusion. The sample therefore does not serve as a counter electrode (as it is the case for dielectric barrier discharge plasma devices) and complications with electrical currents are avoided so that 'safe' applications *in vivo* are possible.

In analogy to the above-mentioned studies on melanoma cells, we observed similar effects on induction of cell apoptosis by higher doses of CAP. Two minutes of CAP treatment initiated about 50% apoptosis in melanoma cancer cell lines – a threshold at which CAP treatment does not cause immediate necrosis but initiates complex cascades of biochemical processes leading to cell death many hours and even days following the treatment. Interestingly, CAP shows very high efficiency in inducing apoptosis in melanoma cells, compared with other anti-tumor agents like disulfiram, indirubin derivative or the isoflavone genistein (24–27), where usually a maximum of approximately 30% apoptotic cells was achieved. These results suggest CAP as a novel medical application with high potential for melanoma treatment.

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Our research is the first to determine the effects on cell inactivation without induction of apoptosis using lower CAP doses. 1 min of CAP treatment is not enough to induce apoptosis using the SMD technology. Effects of lower doses are interesting with regard to tumor therapy as here sub-lethal doses also result in therapeutic effects without damaging the surrounding healthy tissue. We observed that melanoma cells, treated by CAP at doses significantly below those required for inducing cell apoptosis, 'survive' the treatment going into senescence posttreatment and immediately stop proliferation by endogenous signalling cascades. Cellular senescence, a permanent cell cycle arrest, is considered a safeguard mechanism that normally prevents aged or abnormal cells from further expansion. Although the term 'replicative senescence' stands for the widely accepted model of a terminal growth arrest due to telomere attrition, the significance of 'oncogene-inducible senescence' remained an issue of debate over the years. A number of recent studies show the effect of this acute and telomere-independent form of senescence as a tumor-protective, fail-safe mechanism *in vivo* that shares conceptual and possibly therapeutic similarities with the genetically encoded apoptosis machinery (28–30). Interference at this point could potentially be an intriguing approach for cancer treatment, especially, if by manipulation of the CAP parameters, the treatment can be made selective to cancerous cells over healthy cells.

In melanoma, senescence is known to play an important role preventing tumor development. A BRAF-induced senescent growth arrest seems to limit melanomagenesis in humans (31). Work by Daniel Peeper and colleagues addressed the phenomenon that nevi (benign tumors of cutaneous melanocytes), frequently carrying $BRAF^{V600E}$ mutation, may lack any apparent proliferative activity often for decades before lesions in a small subset of cases eventually progress into a malignant melanoma (32,33). Here, we present that using moderate CAP doses this senescence can be re-induced in tumor cells. Whether this effect is reversible in long-term culture still needs to be studied. At least using xCELLigence real-time analysis until 90 h no regrowth of Mel Im cells, a CAP-sensitive cell line, was observed.

Important to note is that the sensitivity of the tumor cell lines to CAP therapy is independent of the progress of malignancy. In our study, we observed no correlation between premetastatic and metastatic cell lines. Additionally, we could show that normal melanocytes are less sensitive to CAP therapy in comparison with tumor cells derived from primary or metastatic melanomas.

To our knowledge, induction of senescence by cold atmospheric plasma was not described before but seems to be an important mechanism to inactivate tumor cells under moderate CAP conditions. It would be interesting to know whether the induction of senescence in tumor cells is a general mechanism induced by CAP under moderate conditions or depends on the new plasma device using the SMD technology. Additionally, further studies must focus on the mechanism how CAP indicates cellular senescence.

In summary, in this study, we revealed that SMD plasma technology is a promising new tool with potent anti-tumor abilities. Higher doses led to strong induction of cellular apoptosis of tumor cells, whereas sub-lethal doses resulted in induction of senescence and thereby stopped tumor cell growth.

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Author contribution

SA, AKB analysed data and wrote the paper, EW, SA performed experiments, Y-FL, TS, HM, GM, JZ contributed plasma knowledge, SK, JZ critically revised the work and AKB projected and designed the study.

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Conflict of interests

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Cell morphology and cell number after 2 min of CAP treatment.

Figure S2. Quantification of the apoptosis protein array.

Figure S3. Cell behaviour after 1 min of CAP treatment.

Figure S4. Determination of senescence in melanocytes and melanoma cells after 1 min of CAP treatment.

Table S1. Characteristics of the used CAP device.

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