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Inhibition of Cell Growth of the Prostate Cancer Cell Model LNCaP by Cold Atmospheric Plasma

MARTIN WEISS[1], DENIS GÜMBEL[2], NADINE GELBRICH[2], LARS-OVE BRANDENBURG[3], ROBERT MANDELKOW[1], UWE ZIMMERMANN[1], PATRICK ZIEGLER[4], MARTIN BURCHARDT[1] and MATTHIAS B. STOPE[1]

[1]*Department of Urology and* [2]*Department of Trauma and Orthopedic Surgery, University Medicine Greifswald, Greifswald, Germany;*
[3]*Department of Anatomy and Cell Biology and* [4]*Institute for Occupational and Social Medicine, RWTH Aachen Universtity, Aachen, Germany*

Abstract. *Backround/Aim: Physical plasmas are ionized gases containing several biologically-reactive factors that yet exert their anti-microbial and anti-proliferative effects in fields of surface sterilisation, de-contamination and wound healing. Materials and Methods: Cold atmospheric plasma (CAP) was generated via the atmospheric pressure plasma jet kINPen09. Apoptotic effects of CAP treatment on the human epithelial prostate cancer cell line LNCaP as a cell culture model for malignant tumor tissue was analyzed by cell counting, western blot and quantitative reverse transcription-polymerase chain reaction analysis. Results: LNCaP cells exhibited significantly reduced cell growth following CAP treatment. We show that most probably the induction of apoptosis is the terminus of CAP treatment illustrated by the pro-apoptotic modulation of p53, p21, caspase-3, Bax, and survivin, as well as morphological changes of cell architecture. Conclusion: Our in vitro study offers first indicatory results for molecular response mechanisms after CAP treatment in a suitable LNCaP cell model.*

Cold atmospheric plasma (CAP) recently offered promising possibilities for its application in decontamination of surfaces, food and wound sites (1-3). Especially in dermatology and dentistry CAP showed notable anti-microbial effects while being non-destructive to adjacent vital tissue and effectively inactivating various microbes and parasites (2, 4, 5). Its biological activity is based on diverse reactive factors involving ions, electrons, reactive oxygen (ROS) and nitrogen species (RNS) as well as ultraviolet radiation, visible and infra-red radiation which arise by ionisation of the carrier gas (6, 7). Due to the characteristic of electrons in an electric field heating-up much faster compared to ions, CAP is characterized by ambient temperature and treatment of biological tissues and cells becomes feasible (5). Besides, recent studies suggested also anti-neoplastic effects of CAP treatment on several tumors (8-10), however, the underlying cellular mechanisms are yet unclear, especially the modality of CAP-dependent cell death being necrotic or apoptosis-controlled.

Prostate cancer (PC) represents one of the most diagnosed malignant diseases and remains the second-leading cause of tumor-associated deaths in male in the Western hemisphere (11). The PC cell line LNCaP is a suitable model to contribute to our understanding of CAP-driven tumor effects.

Programmed apoptotic cell death takes an important place in carcinogenesis and cancer treatment. Targeted-therapy is the principle of much new anticancer therapeutics which specifically induce apoptosis in cancer cells. As apoptosis is a relatively well-investigated cellular mechanism, several key factors were repeatedly identified to play a dominant role in a gene-directed cross-talk program, *e.g.* p53, p21, Bax, survivin as well as caspases (12-14). In the present *in vitro* study we tempt to draw conclusions on CAP's molecular mode of action by analysis of important apoptosis modulators' expression pattern. Our study strongly evidences apoptotic mechanisms being responsible for CAP-driven attenuation of cancer cell proliferation.

Materials and Methods

Cell culture. The human epithelial PC cell line LNCaP (Cell Lines Service, Eppelheim, Germany) was propagated in RPMI 1640

Correspondence to: Matthias Stope, Department of Urology, University Medicine Greifswald, Ferdinand-Sauerbruch-Straße, 17475 Greifswald, Germany. Tel: +49 38348680436, Fax: +49 38348680435, e-mail: matthias.stope@uni-greifswald.de

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medium with 10% fetal calf serum and 100 units/ml penicillin/streptomycin (PAN Biotech, Aidenbach, Germany) at 37˚C and 5% CO_2 atmosphere.

Cold atmospheric plasma (CAP) treatment. The generation of CAP using argon as carrier gas was performed by the atmospheric pressure plasma jet kINPen09 (Neoplas, Greifswald, Germany) (Figure 1). For CAP treatment cells were suspended in 500 μl RPMI 1640 medium (PAN Biotech) on an uncoated cell culture plate. The CAP and argon gas control treatment was performed for 10 sec. After CAP treatment cells were immediately transferred to poly-L-lysine (PAN Biotech) coated cell culture plates and were incubated in RPMI 1640 medium for indicated time points. The following kINPen09 setting was used for CAP treatment: Argon gas flow: 3 l/min; supply voltage=65 V DC; frequency: 1.1 MHz; exposure time: 10 sec. Control cells: Argon gas flow: 3 l/min; exposure time: 10 sec.

Proliferation assay. Cellular proliferation was analyzed using a CASY Cell Counter and Analyzer Model TT (Roche Applied Science, Mannheim, Germany). After CAP treatment of 3×10^4 LNCaP cells being suspended in 500 μl RPMI media, cell counting was done in 24-well cell culture plates (1 ml/well). Adherent cells were detached by 0.1% trypsin/0.04% ethylenediaminetetraacetic acid (EDTA) treatment and resuspended in CASYton solution (Roche Applied Science). The number of viable cells was determined in duplicates for each passage. For further experiments 10 nM docetaxel (Sigma Aldrich, Munich, Germany) was added to RPMI medium, respectively.

Light microscopy. All images of unstained cells were taken by a VisiScope IT404 Microscope (VWR International, Darmstadt, Germany) using a 10×/0.25 Plan acromatic IOS objective lens (VWR International).

Western blotting. 6×10^5 LNCaP cells suspended in 500 μl RPMI medium were CAP-treated for 10 sec and seeded onto a 6-well cell culture plate containing 2.5 ml RPMI medium. Adherent cells were detached using 0.1% trypsin/0.04% EDTA and lysed in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM K_2HPO_4, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.05% sodium dodecysulfate, 1 mM Na_3VO_4, 20 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM 2-phosphoglycerate, and complete protease inhibitor cocktail from Roche Applied Science] at indicated time points. Protein concentrations were determined using Bradford solution (Bio-Rad, München, Germany). Equal amounts of protein (50-100 μg) were separated by SDS-PAGE Mini-Protean system (Bio-Rad) and transferred onto a Protean nitrocellulose membrane (Whatman, Dassel, Germany) using Trans-Blot SD semi-dry transfer cell (Bio-Rad). Membranes were blocked with 1× Roti-Block (Carl Roth, Karlsruhe, Germany). Proteins of interest were detected by specific antibodies directed against p53, phospho-p53 (P-p53), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Danvers, MA, USA). Protein signals were visualized with the ChemiDoc system (Bio-Rad) using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) and quantified using Image Lab 3.0 (Bio-Rad) software.

Quantitative reverse transcription-polymerase chain reaction. 6×10^5LNCaP cells suspended in 500 μl RPMI medium were CAP treated for 10 sec and seeded onto a 6-well cell culture plate containing 2.5 ml RPMI medium. Adherent cells were detached by 0.1% trypsin/0.04% EDTA and RNA was isolated by peqGOLD TriFast (Peqlab Biotechnology, Erlangen, Germany) according to manufacturer's instructions. Reverse transcription was performed using 1 μg of total RNA (RevertAid First Strand Synthesis Kit, Fermentas, Vilnius, Lithuania). Increasing fluorescence of SYBR dye (SensiMix SYBR Kit, Bioline, London, UK) was monitored in real-time in a CFX96 Real-Time PCR Detection System (Bio-Rad) applying the following PCR primers: caspase-3 (caspase-3 forward 5'-GCTC CTAGCGGATGGGTGCT-3', caspase-3 reverse 5'-GATTCCAAGGC GACGCCAAC-3'), p21 (p21 forward 5'-TGGAGACTCTCAG GGTCGAAA-3', p21 reverse 5'-GGCGTTTGGAGTGGTAGAAATC-3'), Bax (Bax forward 5'-TCCCCCCGAGAGGTCTTTT-3', Bax reverse 5'-CGGCCCCAGTTGAAGTTG-3'), survivin (survivin forward 5'-TGCCCCGACGTTGCC-3', survivin reverse 5'-CAGTTCTTGAATGTAGAGATGCGGT-3') and ribosomal protein large P0 as reference gene (RPLP0 forward 5'-CAATGGCAG CATCTACAACC-3', RPLP0 reverse 5'-ACTCTTCCTTGGCT TCAACC-3'). For quantification, signals were standardized to RPLP0 as reference.

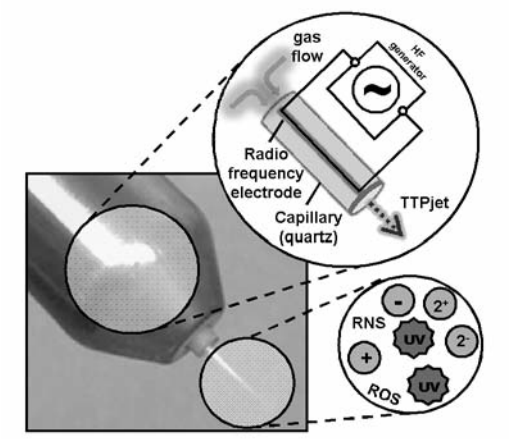

Figure 1. *The cold atmospheric plasma (CAP) source. Plasma jet kINPen09 (Neoplas GmbH, Greifswald, Germany): schematic reconstruction and composition of physical plasma.*

Statistics. Statistical analysis was carried out using the unpaired Student's *t*-test. Results of $p<0.05$ were considered statistically significant. Data are given as mean±SD.

Results

Treatment with CAP attenuated cellular proliferation of human PC cells. In the present study we analyzed the effects of a single short CAP treatment (10 sec) on proliferation of human PC cell line LNCaP. By cell counting the total cell number was determined over a period of 120 h (Figure 2A). CAP treatment caused significant reductions in cell numbers of the observed cell lines LNCaP (Figure 2A; 4 h: 1.33-fold, $p=0.0089$; 24 h: 2.05-fold, $p<0.001$; 48 h: 3.11-fold, $p<0.001$; 72 h: 5.89-fold, $p<0.001$; 96 h: 7.74-fold, $p<0.001$; 120 h:

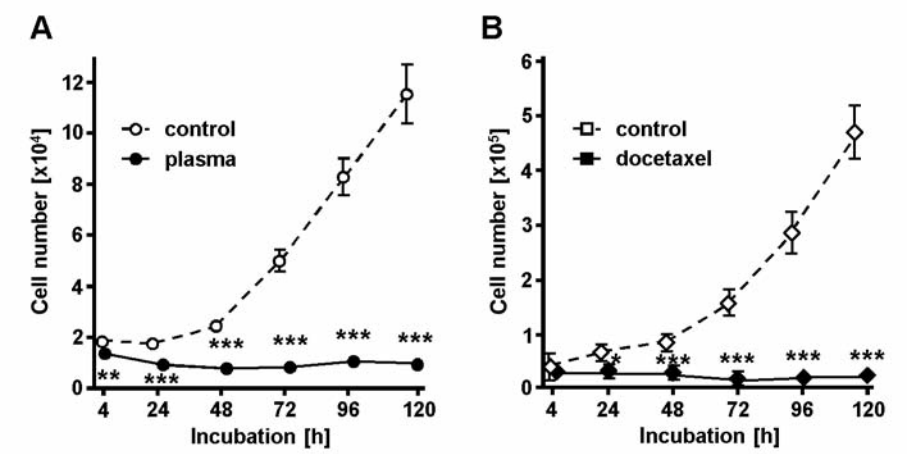

Figure 2. *Cold atmospheric plasma (CAP) inhibits cellular growth of human prostate cancer cells. Cells were counted using a CASY Cell Counter and Analyzer Modell TT at indicated time points following CAP treatment for 10 sec. Controls were treated with argon for 10 sec. (A) Viable cell number of LNCaP cells treated with CAP revealed significantly decreased cell numbers compared to controls. (B) LNCaP cells incubated with 10 nM docetaxel showed significantly inhibited cell growth, comparable to CAP treatment. Results are expressed as the mean±SD of cell count. *p<0.05; ***p<0.001, as determined by the Student's t-test.*

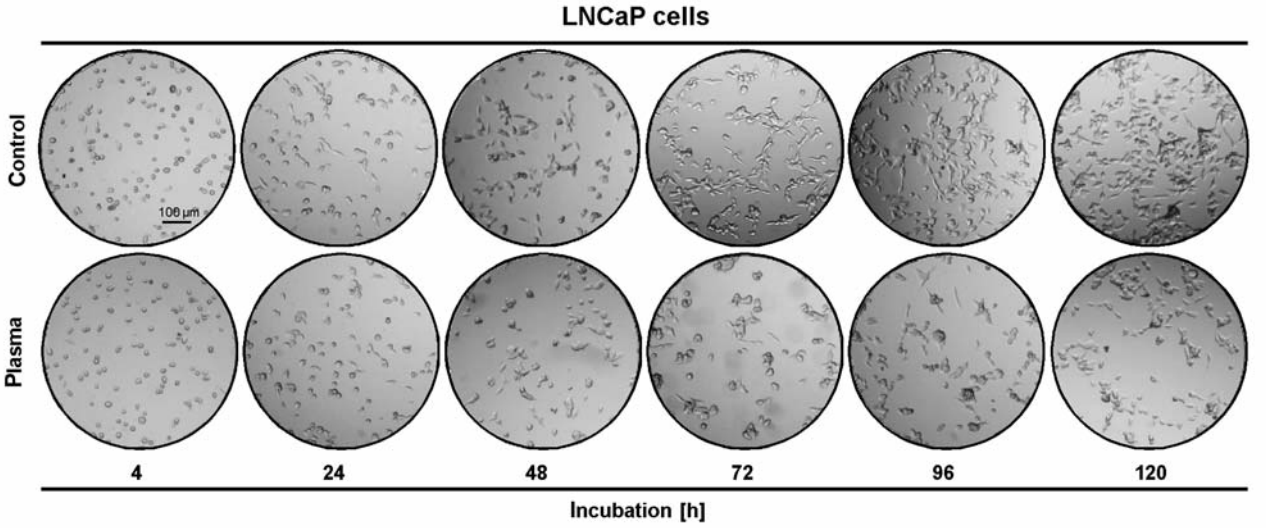

Figure 3. *Cold atmospheric plasma (CAP) treatment induces morphological chances in LNCaP cells. Light microscopic analysis of CAP-treated LNCaP cells compared to argon-treated controls after indicated time points. LNCaP control cells (top) show the known morphology and regular cell growth. CAP-treated LNCaP cells fail to develop the regular cell morphology and remain at a low cell number. Bar: 100 µm.*

11.54-fold, $p<0.001$) compared to argon-treated controls. The application of CAP on PC cell models is a preliminary step prior to *in vivo* studies. Any new potential method for oncological treatment must further successfully compete with established treatment regimen. To specify the cytostatic potency of CAP treatment, we compared the CAP effect on LNCaP cells with the anti-proliferative compound docetaxel, a first line drug in treatment of castration-resistant PC. Incubation of LNCaP cells with 10 nM docetaxel for 120 h showed a similar suppressive effect on cell growth compared to CAP treatment (Figure 2B; 24 h: 1.31-fold, $p=0.0293$; 48 h: 2.99-fold, $p=0.0005$; 72 h: 8.92-fold, $p<0.0001$; 96 h: 19.30-fold, $p<0.0001$; 120 h: 35.71-fold, $p<0.0001$).

CAP treatment induced severe morphological changes of LNCaP cells most reminiscent of apoptotic cell death. Light

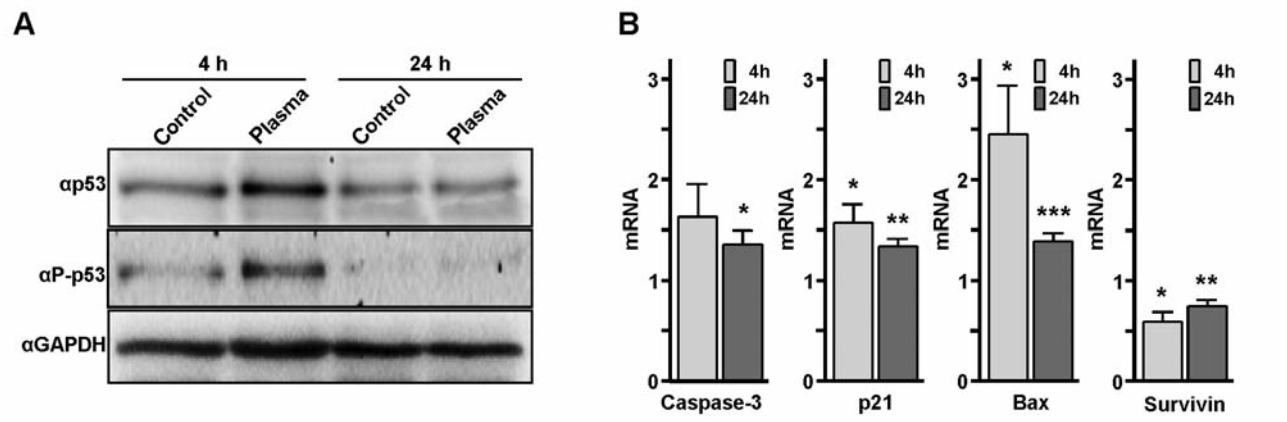

Figure 4. *Modulated mRNA levels and protein expression of pro- and anti-apoptotic proteins following cold atmospheric plasma (CAP) exposure. LNCaP cells were treated with CAP for 10 sec and were cultured for 4 and 24 h. Argon-treated cells were used as control. Cells were harvested, lysed and analyzed by western blot (A) and quantitative RT-PCR analysis (B). (A) Representative western blot of p53 and phosphorylated p53 (P-p53) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as loading control. p53 expression level and p53 protein phosphorylation (P-p53) increased shortly after CAP treatment. This effect was abrogated within 24 h. (B) mRNA of Caspase-3, p21, Bax, and Survivin of CAP treated LNCaP cells compared to controls. CAP exposure led to a significant increase of pro-apoptotic caspase-3, p21 and Bax transcripts, whereas mRNA levels of anti-apoptotic survivin were significantly decreased. Data were standardized to untreated control cells and are expressed as the mean±SD. *p<0.05, **p<0.01, ***p<0.001, as determined by the Student's t-test.*

microscopy analyses revealed that CAP treated LNCaP cells exhibit considerable changes in morphology and cell growth compared to control cells. Beside a reduced cell number and visible cell debris, CAP exposed cells were less widespread, hardly developed the typical polyedric cell shape, and revealed a light microscopic structural appearance of cells underlying apoptotic DNA fragmentation (Figure 3).

LNCaP cells exhibit altered regulation of apoptotic factors in response to CAP treatment. To investigate whether the reduction of cell numbers after CAP treatment is the result of apoptotic cell death, we tempted to identify CAP-induced apoptosis-related factors. Total protein amounts of p53 and P-p53 were investigated by western blot analysis (Figure 4A). mRNA amount of caspase-3, p21, Bax and survivin were analyzed using quantitative real time PCR and were standardized to untreated control cells (Figure 4B). Expression of p53 was found to be up-regulated exclusively 4 h after the CAP treatment compared to argon-treated control cells. No significant differences were determined after 24 h (Figure 4A; αp53). Besides the CAP-driven induction of p53 we observed a strong p53 phosphorylation 4 h after CAP treatment (Figure 4A; αP-p53). Notably, this transient phosphorylation was almost completely reversed after 24 h following the CAP treatment. Moreover, 10 sec of CAP treatment caused significant mRNA induction of caspase-3 (Figure 4B; casp3; 4 h: 1.63-fold; 24 h: 1.36-fold, p=0.0355), p21 (Figure 4B; 4 h: 1.57-fold, p=0.0359; 24 h: 1.34-fold, p=0.0099) and Bax (Figure 4C; 4 h: 2.45-fold, p=0.0355; 24 h: 1.39-fold, p=0.0077). Accordingly, mRNA-levels of the known anti-apoptotic protein survivin were significantly reduced at indicated time points (Figure 4B; 4 h: 1.40-fold reduction, p=0.0139; 24 h: 1.24-fold reduction, p=0.0087), confirming our assumption of CAP being a potent trigger for apoptotis.

Discussion

In the present study we investigated the impact of CAP on LNCaP cells. We could demonstrate a significant attenuation of cellular proliferation after CAP treatment of LNCaP cells (Figure 2). Light microscopy analysis of CAP treated LNCaP cells revealed no signs of necrotic cell lysis, however, further examinations are required to clarify morphological changes following CAP treatment. We observed that cancer cells survived at low but constant levels and actually increased towards the experimental endpoint (Figure 3). Moreover, we ascertained a stimulated p53 activity after CAP treatment as shown by induced expression of total p53 as well as by elevated levels of phosphorylated p53 protein (Figure 4A). Activated p53, as it is commonly seen as guardian of the genome and oncogenes, can either induce DNA-repair or lead to apoptotic cell death. This tempted us to examine further factors of intrinsic apoptosis pathways: the mRNA levels of the pro-apoptotic factors caspase-3, p21 and Bax (15) were significantly elevated, whereas mRNA transcripts of the anti-apoptotic factor survivin were decreased as illustrated in Figure 4B. Interestingly, survivin as an inhibitor of apoptotic proteins, was reported to be over-expressed in

many tumors and being proposed as link to cell proliferation and cell death (16). Depending on whether survivin was expressed or not, cancer cells were resistant to many apoptotic stimuli (17) or, as in case of survivin shut-off, lapsed to spontaneous apoptosis and increased sensitivity to apoptotic stimuli (16). In this study, p53 protein expression and phosphorylation exhibited a conspicuous reduction of CAP effects 24 h following CAP treatment. This indicates very early and transient cellular processes mediated by CAP.

In summary, there exists strong evidence for apoptotic cell death following CAP treatment. Also in case of other solid cancer entities, several studies have shown CAP-induced expression of apoptotic factors. Recently, Arndt *et al*. obtained information on enhanced p53 phosphorylation, as well as induction of caspase-3 and p21 in melanoma cells (8). However, to the best of our knowledge, there are currently no other studies with which the effects of CAP concerning the altered expression levels of p53, Bax and survivin could be compared. In contrast to our data some studies reported necrotic CAP effects, mostly depending on power and duration of CAP exposure (18, 19). Thereby, exposure times exceeding 10 sec and powers higher than 0.2 W resulted in necrotic cell damage and subsequent release of intracellular content. The caveat whether CAPs' anti-proliferative effect on cancer cells occurs by necrosis or apoptosis is challenging, especially because of a variety of different plasma devices, parameters and tissues that have been used in most of the CAP studies.

Adding the data from the present study and in line with previous reports, we conclude that apoptosis must be the predominant molecular mechanism behind CAP-driven cancer cell depletion.

One limitation of our study is its restriction to *in vitro* methodology and the use of a single cell line only. However, the LNCaP cell line is a well-established *in vitro* model for PC, which if only due to its wide distribution yields a great reliability and validity.

Recently, CAP offered promising anti-neoplastic effects in other cancer entities such as pancreatic and lung cancer as well as melanoma (6, 8, 20-22). Some of these *in vitro* studies were already confirmed *in vivo* by nude mice models (23). Our results provide a basis for a better understanding of CAP-induced mechanisms in PC cells and definitely warrant further *in vivo* experiments.

Conflicts of Interests

The Authors have declared that no competing interests exist.

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