Effects of Cold Atmospheric Plasma on the Expression of Chemokines, Growth Factors, TNF Superfamily Members, Interleukins, and Cytokines in Human Osteosarcoma Cells

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Abstract. *Background/Aim:* Therapeutic options for osteosarcoma (OS) are still limited. Cold atmospheric plasma (CAP) leads to inhibition of tumor growth and metastasis, but underlying mechanisms are not fully understood. The aim of this study was to investigate CAP-induced changes in cytokine expression in OS cells. *Materials and Methods:* OS cell lines (U2-OS, MNNG/HOS) were treated with CAP and administered to an RT2 Profiler PCR Array (Qiagen, Hilden, Germany) detecting 84 chemokines, growth factors, TNF superfamily members, interleukins, and cytokines. *Results:* The analyses showed that 15 factors (C5, CCL5, CNTF, CSF1, CSF3, CXCL1, IL-1A, IL-1B, IL-18, IL-22, IL23A, MSTN, NODAL, TGFβ2, THPO) were induced, but only one factor (VEGFA) was suppressed after CAP treatment. *Conclusion:* No extensive systemic cell response with presumably far-reaching consequences for neighboring cells was detectable after CAP treatment. Since the antitumoral effect of CAP on OS cells has already been demonstrated, intraoperative treatment with CAP represents a promising and systemic safe option for the therapy of OS.

Osteosarcoma (OS) is the most common bone cancer in humans and at initial diagnosis, metastases are present in about 20 % of all cases. Standard therapy includes radical surgical resection and chemotherapy, but due to strong toxic effects, new treatment options are urgently needed (1). Currently, there is a growing discussion about expanding the oncological therapy spectrum and treat with cold atmospheric plasma (CAP) (2, 3). CAP represents a highly reactive ionized gas containing radicals, ions, photons, and electromagnetic rays. Its biological effects are primarily mediated by reactive oxygen and nitrogen species (RONS) such as singlet oxygen, superoxide, ozone, hydroxyl radicals, nitrogen radicals, nitric oxide, nitrogen dioxide, peroxynitrite, and hydrogen peroxide (4). Due to its low, almost body-warm temperature, CAP is ideally suited for medical applications and has been used for many years in the treatment of skin diseases and chronic wounds (5). In the context of oncological therapy, the *in vitro* effect of CAP treatment has already been demonstrated for numerous tumor entities including melanoma (6), prostate cancer (7), glioblastoma (8), pancreatic cancer (9), head and neck cancer (10), colon cancer (11), lung cancer (12), leukemia (13), and gastric cancer (14). Initial preliminary *in vivo* studies have shown the antitumoral effect of CAP also for pancreatic cancer (14), melanoma (15), ovarian cancer (16), breast cancer (17), and colon cancer (18). The molecular and cellular mechanisms leading to an inactivation of malignant cells, however, are still poorly investigated and largely unclear.

In OS cells, inhibition of cell growth after CAP treatment has been demonstrated. This was due to the activation of redox signalling cascades, which affected mitochondria's

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Table I. Human Cytokines & Chemokines RT2 Profiler PCR Array (Qiagen). Abbreviation (symbol) and description of all 84 cytokines analyzed by the PCR array in this study. The column "detectable" indicates whether the factor was detected (+) or not detected (–).

Number	Symbol	Description	Detectable
Chemokines			
1	C5	Complement component 5	+
2	CCL1	(C-C motif) ligand 1	–
3	CCL2	(C-C motif) ligand 2	–
4	CCL3	(C-C motif) ligand 3	–
5	CCL5	(C-C motif) ligand 5	+
6	CCL7	(C-C motif) ligand 7	–
7	CCL8	(C-C motif) ligand 8	–
8	CCL11	(C-C motif) ligand 11	–
9	CCL13	(C-C motif) ligand 13	–
10	CCL17	(C-C motif) ligand 17	–
11	CCL18	(C-C motif) ligand 18	–
12	CCL19	(C-C motif) ligand 19	–
13	CCL20	(C-C motif) ligand 20	–
14	CCL21	(C-C motif) ligand 21	–
15	CCL22	(C-C motif) ligand 22	–
16	CCL24	(C-C motif) ligand 24	–
17	CXCL1	Chemokine (C-X-C motif) ligand 1	+
18	CXCL2	Chemokine (C-X-C motif) ligand 2	–
19	CXCL5	Chemokine (C-X-C motif) ligand 5	–
20	CXCL9	Chemokine (C-X-C motif) ligand 9	–
21	CXCL10	Chemokine (C-X-C motif) ligand 10	–
22	CXCL11	Chemokine (C-X-C motif) ligand 11	–
23	CXCL12	Chemokine (C-X-C motif) ligand 12	–
24	CXCL13	Chemokine (C-X-C motif) ligand 13	–
25	CXCL16	Chemokine (C-X-C motif) ligand 16	–
26	CX3CL1	Chemokine (C-X3-C motif) ligand 1	–
27	GPI/AMF	Glucose-6-phosphate isomerase	–
28	IFNA2	Interferon, alpha 2	–
29	MIF	Macrophage migration inhibitory factor	–
30	PPBP	Proplatelet basic protein (chemokine (C-X-C motif) ligand 7)	–
31	SPP1	Secreted phosphoprotein 1	–
32	XCL1	Chemokine (C motif) ligand 1	–
Growth factors			
33	BMP2	Bone morphogenetic protein 2	–
34	BMP4	Bone morphogenetic protein 4	–
35	BMP6	Bone morphogenetic protein 6	–
36	BMP7	Bone morphogenetic protein 7	–
37	CNTF	Ciliary neurotrophic factor	+
38	CSF1	Colony stimulating factor 1 (macrophage)	+
39	CSF2	Colony stimulating factor 2 (macrophage)	–
40	CSF3	Colony stimulating factor 3 (macrophage)	+
41	LIF	Leukemia inhibitory factor (cholinergic differentiation factor)	–
42	MSTN	Myostatin	+
43	NODAL	Nodal growth differentiation factor	+
44	OSM	Oncostatin M	–
45	TGFB2	Transforming growth factor, beta 2	+
46	THPO	Thrombopoietin	+
47	VEGFA	Vascular endothelial growth factor A	+
TNF superfamily			
48	CD40LG	CD40 ligand	–
49	FASLG	Fas ligand (TNF superfamily, member 6)	–
50	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	–
51	LTB	Lymphotoxin beta (TNF superfamily, member 3)	–
52	TNF	Tumor necrosis factor	–
53	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	–
54	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	–

Table I. Continued

Table I. *Continued*

Number	Symbol	Description	Detectable
55	TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11	–
56	TNFSF13B	Tumor necrosis factor (ligand) superfamily, member 13b	–
		Interleukins	
57	IL1A	Interleukin 1, alpha	+
58	IL1B	Interleukin 1, beta	+
59	IL1RN	Interleukin 1 receptor antagonist	–
60	IL2	Interleukin 2	–
61	IL3	Interleukin 3 (colony-stimulating factor, multiple)	–
62	IL4	Interleukin 4	–
63	IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	–
64	IL6	Interleukin 6 (interferon, beta 2)	–
65	IL7	Interleukin 7	–
66	IL8	Interleukin 8	–
67	IL9	Interleukin 9	–
68	IL10	Interleukin 10	–
69	IL11	Interleukin 11	–
70	IL12A	Interleukin 12A	–
71	IL12B	Interleukin 12B	–
72	IL13	Interleukin 13	–
73	IL15	Interleukin 15	–
74	IL16	Interleukin 16	–
75	IL17A	Interleukin 17A	–
76	IL17F	Interleukin 17F	–
77	IL18	Interleukin 18 (interferon-gamma-inducing factor)	+
78	IL21	Interleukin 21	–
79	IL23A	Interleukin 23, alpha subunit p19	+
80	IL22	Interleukin 22	+
81	IL24	Interleukin 24	–
82	IL27	Interleukin 27	–
		Cytokines	
83	ADIPOQ	Adiponectin, C1Q and collagen domain containing	–
84	IFNG	Interferon, gamma	–

functionality and subsequently led to the induction of apoptosis (19, 20).

Furthermore, indirect effects can also be assumed, which regulate adjacent cells via the secretion of soluble factors and result in an amplification of the CAP effect. Treatment with CAP has been shown to alter the expression of several thousand genes and may affect important factors such as growth factors, cytokines, interleukins, and members of the tumor necrosis factor (TNF) superfamily. As a result, important cell responses such as proliferation, inflammation, and immune response can be modulated in cancer cells as well as adjacent non-malignant cells (21, 22).

The aim of this study was to investigate changes in cytokine and chemokine expression in OS cells treated with CAP. The pattern of these factors would significantly influence the behavior of both tumor cells as well as cells of the tumor microenvironment. This would be of particular interest with regard to the biological effect of CAP, especially in therapy approaches where CAP is applied to OS patients.

Materials and Methods

Cell culture. The human OS cell lines U2-OS and MNNG/HOS (purchased from the American Type Culture Collection, Manassas, VA, USA) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 1.0 g/l glucose supplemented with 10% foetal bovine serum, 1 mM sodium pyruvate, and 1% penicillin/streptomycin (all PAN Biotech, Aidenbach, Germany) in a humidified atmosphere at 5% CO₂ and 37°C. A total of 4×10⁶ cells were seeded on an uncoated cell culture plate. After 4 days, cells were washed twice in phosphate-buffered saline (PBS) and detached using 0.1% trypsin/0.04% ethylenediaminetetra-acetic acid (EDTA) and resuspended in DMEM.

CAP treatment. The atmospheric plasma jet kINPen MED (Neoplas Tools, Greifswald, Germany) was utilized for CAP generation (carrier gas: argon, gas flow: 3 l/min; supply voltage=65 V DC; frequency: 1.1 MHz). A total of 5×10⁵ (U2-OS) and 5×10⁵ (MNNG/HOS) cells were suspended in 500 µl DMEM and treated for 10 s in suspension following a standard meandering pattern. After CAP treatment, cells were immediately transferred to poly-L-lysine (PAN Biotech)-coated 24-well cell culture plates and

Figure 1. Relative expression of differentially expressed factors in CAP-treated MNNG/HOS cells. CAP-treated cells were administered to an RT² Profiler PCR Arrays (Qiagen, Hilden, Germany) detecting 84 factors belonging to the groups of chemokines, growth factors, TNF superfamily members, interleukins, and cytokines.

incubated in DMEM with for 24 h (in a humidified atmosphere at 5% CO₂ and 37°C).

Control group. A control group total of 5×10⁵ (both U2-OS and MNNG/HOS) cells were suspended in 500 µl DMEM and treated for 10 sec only with Argon gas in suspension following a standard meandering pattern. After control treatment, cells were transferred and incubated similar to the CAP treated cells.

RNA preparation with peqGOLD TriFast™. Cells were washed with 3.0 ml PBS, harvested and total RNA was isolated with 500 µl peqGOLD TriFast™ reagent (Peqlab Biotechnology, Erlangen, Germany) as described by the supplier. Isolated RNA was quantified spectrophotometrically with the NanoDropND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

Quantitative real-time polymerase chain reaction-based array. RT² Profiler PCR Arrays (Qiagen, Hilden, Germany) were used according to the supplier's instructions to monitor the expression of 84 genes of both OS cell lines for cytokine profiling (Table I). The ΔΔCt method was used to determine the relative quantity of gene expression. Microarray data was normalized against reference genes and CAP treated approaches were compared to control treated approaches by calculating the ΔΔCt for each gene of interest.

Statistics. For data evaluation, the graphics and statistics the Graph Pad Prism V5.01 software (GraphPad Software, La Jolla, CA, USA) was used. The values of two independent passages with both cell lines were averaged and displayed as fold expression. Due to this small number of runs, the standard deviation and the calculation of significance were omitted.

Results

The quantitative PCR-based analysis included a total of 84 factors belonging to the groups of chemokines, growth factors, TNF superfamily members, interleukins, and cytokines. For the analysis, a protein induction higher than the double (≥2.0) and a reduction of expression to at least half (≤-2.0) were defined as cut-off values. Compared to control cells, 8 of these factors in MNNG/HOS cells (Figure 1) and 10 factors in U2-OS cells (Figure 2) were differentially regulated following CAP treatment.

In case of MNNG/HOS cells (Figure 1), 1 chemokine ((C-X-C motif) ligand 1 (CXCL1; 16.8-fold)), 2 interleukins (interleukin-22 (IL-22; 267.5-fold) and interleukin 23 submit alpha (IL23A; 38.9-fold)), and 4 growth factors (colony stimulating factor 1 (CSF1; 2.5-fold), colony stimulating factor 3 (CSF3; 202.5-fold), myostatin (MSTN; 700.0-fold), and transforming growth factor β2 (TGFβ2; 7.3-fold)) were induced by CAP treatment. Only the expression of a single factor (vascular endothelial growth factor-A (VEGFA; -2.7-fold)) was attenuated after CAP treatment compared to control treated cells.

The treatment of U2-OS cells with CAP (Figure 2) led to the induction of 3 chemokines (complement component 5 (C5; 3.2-fold), chemokine (C-C motif) ligand 5 (CCL5; 2.3-fold), and chemokine (C-X-C motif) ligand 1 (CXCL1; 2.6-fold)), 4 interleukins (interleukin 1 alpha (IL-1A; 6.0-fold),

Figure 2. Relative expression of differentially expressed factors in CAP-treated U2-OS cells. CAP-treated cells were administered to an RT² Profiler PCR Arrays (Qiagen) detecting 84 factors belonging to the groups of chemokines, growth factors, TNF superfamily members, interleukins, and cytokines.

interleukin 1 beta (IL-1B; 3.9-fold), interleukin-18 (IL-18; 2.3-fold), and interleukin-22 (IL-22; 98,430.9-fold)), and 3 growth factors (ciliary neurotrophic factor (CNTF; 2.8-fold), Nodal growth differentiation factor (NODAL; 9.5-fold), and thrombopoietin (THPO; 3.5-fold)).

Discussion

This study showed, for the first time, the modulation of gene expression after CAP treatment in OS cells. The research focused on cellular factors which, as secretory factors, regulate proliferative, immunological, and inflammatory processes in the microenvironment of tumors. However, the analyses were limited to the expression rate of the factors on the mRNA level. The further processing and in particular the secretion of the molecules into the extracellular space were not considered. In this respect, this is an important limitation of this study.

In the two OS cell lines used, 8 (MNNG/HOS cells) and 10 (U2-OS cells) factors were differentially expressed. Two of these factors were identified as CAP modulated in both cell lines. These were chemokine CXCL1 and interleukin IL-22, which were significantly upregulated in both OS cell lines by CAP treatment. While the induction of the CXCL1 expression by 16.8-fold (MNNG/HOS cells) and 2.6-fold (U2-OS cells) was comparatively moderate, IL-22 was increased by several hundred to several thousand times in both OS lines (MNNG/HOS: 267.5-fold; U2-OS: 98,430.9-fold). CXCL1 as well as IL-22 possess pro-oncogenic properties in the tumor biological context. CXCL1 controls

cell growth, epithelial-stroma interactions, and cell motility and has been described as a negative prognostic biomarker in some tumor entities including skin, colon, and prostate cancer (23-25). In OS cells, expression of chemokines has been generally defined as biomarker of tumor progression (26). The tumor biological function of IL-22 is similar. It correlates with tumor progression in gastric, colorectal, and pancreatic cancer (25, 27, 28) and determines the epithelial-to-mesenchymal transformation of breast cancer cells (29). For both factors, CXCL1 and IL-22, no data on their role in the development of OS are, so far, available. However, this study suggests that they may have pro-oncogenic properties.

The remaining 14 factors differentially expressed after CAP treatment could only be detected in one of the two OS cell lines. This is presumably due to the high individuality of each tumor, which occurs frequently in the clinical situation. Such differences on the molecular and cellular level could also explain the individual response and the individual progress of OS. If examined in tumor cells, pro-oncogenic functions can also be attributed to these 14 factors. CSF-1, CSF-3, and NODAL for example, have been associated with progression in breast, and prostate cancer (30, 31). The four groups of chemokines (CXC, CC, CX3C, and C chemokine ligands) play central roles in tumorigenesis, including angiogenesis, and are therefore also discussed as targets for anticancer therapies (32). Growth factors are also involved in growth and progression in many tumor types (33). However, growth factors of the TGF β

family play a special role here, since they can have both pro-oncogenic and tumor suppressive functions. These depend on the tumor stage and play an important role in therapy resistance, for example (34). Consequently, the tumor biological role of CAP-induced TGF β 2 in MNNG/HOS cells cannot be determined beyond doubt.

VEGFA was the only factor whose expression rate was reduced by CAP treatment of MNNG/HOS cells. The VEGF pathway is one of the most important signalling cascades in angiogenic processes and is, therefore, both an important biomarker of tumor progression and a target for anticancer therapy (35). Thus, CAP-mediated repression of VEGFA expression would be an important tumor suppressive mechanism of CAP. This means that in addition to the antiproliferative effects shown *in vitro* and *in vivo*, CAP would also suppress blood supply to tumor cells, which is very important for clinical use in patients.

In summary, it can be said that the treatment of OS cells with CAP leads to induction of a number of growth-promoting and pro-oncogenic factors. This should probably be understood as part of a stress response of cancer cells. However, in *in vitro* experiments with the cell lines used, CAP treatment led to significantly suppressed cell growth (19, 20), so that this effect may be negligible in a potential therapeutic application of CAP. On the other hand, CAP also leads to the induction of TGF β 2, a potentially tumor suppressive factor in OS, and to the suppression of the angiogenic factor VEGFA. Both could have pro-therapeutic effects in the application of CAP. This study also shows that only 16 of the 84 factors examined are modulated by CAP. There was no extensive systemic cell response with presumably far-reaching consequences for neighboring cells. Thus, it can be concluded that intraoperative treatment with CAP may represent a promising option for the future therapy of OS.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

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