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Effect of Reoxygenation on the Hypoxia-Induced Up-Regulation of Serine Protease Inhibitor PAI-1 in Head and Neck Cancer Cells

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Key Words
Urokinase-type plasminogen activator · Plasminogen activator inhibitor-1 · Hypoxia · Reoxygenation · Head and neck cancer

Abstract
In squamous cell carcinoma of the head and neck (SCCHN), hypoxia is considered a crucial physiological modulator for malignant progression, whereby the plasminogen activation system is involved in overlapping functions such as moulding of the extracellular matrix, cell proliferation and signal transduction. Little is known about the effects of reoxygenation on the plasminogen activation system in SCCHN cells. Three human SCCHN cell lines (BHY, CAL27, FaDu) and a non-transformed human fibroblast cell line (VH7) were exposed to hypoxic (<0.5% O_2) conditions for up to 72 h and subsequently reoxygenated at normoxic conditions for 24 h. Urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) protein concentration and former protein activity were determined by ELISA and complex ELISA, respectively. Reoxygenation induced significant changes in cell-associated and secreted PAI-1 protein compared to the normoxic control. Significant increase in cell-associated and secreted uPA protein after reoxygenation was only observed for some of the cell lines. Determination of uPA-PAI-1 complex formation revealed the release of active protein into the cell supernatant. The beneficial role of reoxygenation during radiation therapy is widely accepted. However, reoxygenation does not seem to counteract the effects induced by hypoxia on the plasminogen activation system. Fatally irradiated reoxygenated tumour cells might still produce sufficient amounts of 'harmful' protein and thus initiate a path for invasion and metastasis for surviving tumour cells.

Introduction

Tumour hypoxia is regarded to be a crucial physiological modulator of malignant progression since hypoxic tumours tend to be biologically more aggressive, resistant to radiation therapy, and therefore more likely to recur locally or metastasise [1, 2]. Hence, one of the aims during radiation therapy is to induce reoxygenation of hyp-

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oxic regions within the tumour to increase radiosensitivity and ultimately to obtain higher cell kill [3]. In squamous cell carcinomas of the head and neck (SCCHN), reoxygenation during radiation therapy has been observed. The impact of reoxygenation during therapy, however, is not entirely clear and conflicting results regarding the correlation between reoxygenation and treatment outcome have been reported [4, 5].

Among the many genes influenced by hypoxia is the plasminogen activator inhibitor-1 (PAI-1) [6], a major inhibitor of the serine protease urokinase-type plasminogen activator (uPA). PAI-1 inhibitory activity is stabilised upon binding to the extracellular matrix (ECM) protein vitronectin, a cell adhesion glycoprotein instrumental in tissue turnover, also in cancer. Usually, uPA is synthesised and secreted as an almost inactive proenzyme (pro-uPA) by various normal and cancer cells. Its proteolytic activity is accelerated via cleavage by proteases such as plasmin, and this highly enzymatically active form of uPA (HMW-uPA) is transferred to an even higher state of activity after binding to the specific membrane-associated receptor uPAR (CD87). In turn, HMW-uPA converts the proenzyme plasminogen to plasmin which can either degrade ECM components directly or act indirectly by activating pro-matrix metalloproteinases (MMPs), thus promoting metastatic cell spread [7]. PAI-1 binds to the cell membrane-associated uPAR-uPA complex and the resulting uPAR-uPA-PAI-1 complex is subsequently internalised by the cell, thereby initiating signal transduction and cell proliferation. Together with other members of the plasminogen activation (PA) system, PAI-1 is considered to be an essential regulating factor in tumour invasion and metastasis [8, 9]. Hypoxia, on the other hand, has also been shown to be an instigator of tumour growth, invasion and metastasis by inducing angiogenesis. The resulting migration of endothelial cells necessitates proteolysis of the ECM which is achieved by significantly up-regulating uPA and uPAR expression and thereby enhancing proteolytic activity. However, the response of the members of the PA system to reduced oxygen availability is not a uniform one. Cultivation of human trophoblast cells or bovine endothelial cells under low oxygen concentrations results in an up-regulation of PAI-1 mRNA and secreted PAI-1 protein levels [6, 10], whereas secretion of PAI-1 is decreased in human corneal epithelial cells [11].

In human microvascular endothelial cells cultured under hypoxic conditions, uPA protein secretion was decreased [12]. Likewise, Graham et al. [13], when investigating breast carcinoma cells exposed to hypoxic growth conditions, found that increased uPAR expression was paralleled by higher cell-associated uPA and decreased secreted uPA protein levels. Looking at a further species, Pinsky et al. [14] reported decreased uPA activity in hypoxic murine lung tissue due to decreased uPA mRNA and increased PAI-1 expression. Both uPA and PAI-1 have been correlated to poor clinical outcome in several types of cancer, including breast, stomach and lung cancer as well as cancer of the oral cavity [15–19]. In head and neck tumours, several studies have shown uPA and PAI-1 to be markers of disease-free survival [20, 21]. Although hypoxia and the PA system are both known to influence disease outcome negatively, to date, only Stadler et al. [22] have linked these 2 aspects and successfully demonstrated a correlation between increasing uPA antigen levels and decreasing tumour oxygenation levels in patients with SCCHN.

The present study analysed the influence of various exposure times to prolonged hypoxia (<0.5% O_2) with and without subsequent 24-hour reoxygenation on uPA and PAI-1 protein expression in 3 human cell lines originating from SCCHN. Our focus was centred on PAI-1 due to its key role in tumour invasion and metastasis. We chose an in vitro approach to study the isolated effect of hypoxia and reoxygenation in a standardised environment thereby avoiding interfering effects caused by treatment, such as radiation or chemotherapy.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM; 4,500 mg/l D-glucose, 25 mM HEPES, without sodium pyruvate), calcein acetoxymethyl ester (calcein AM) and AlexaFLUOR 488 goat-anti mouse IgG (both Molecular Probes) were obtained from Invitrogen (Karlsruhe, Germany). Foetal calf serum (FCS) and cell culture plastic ware were supplied by Biochrom AG (Berlin, Germany). Phosphate-buffered saline (PBS), Tween 20, EDTA, Triton X-100, NaCl, saponin, propidium iodide and tris(hydroxymethyl)aminomethane were purchased from Sigma-Aldrich (Taufkirchen, Germany). Anti-human proliferation marker Ki-67 (clone MIB-1) was purchased from Dako (Hamburg, Germany). Protein assay kit was obtained from Pierce Perbio Science (Bonn, Germany). ELISA kits for uPA (Imubind No. 894) and for PAI-1 (Imubind No. 821) were supplied by American Diagnostica Inc. (Stamford, Conn., USA).

Cultivation of Cells under Normoxic and Hypoxic Conditions

The human adherently growing cell lines BHY (ACC 404) and CAL27 (ACC 446) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). FaDu (ATCC HTB-43) and VH-7 cells (human foreskin) were kindly provided by M. Baumann (Dresden, Germany) and

P. Boucamp (Heidelberg, Germany), respectively. All cell lines were cultivated in DMEM supplemented with 10% FCS. Cell cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C (later referred to as standard conditions). Cells from passages 10–25 were used for all experiments. Cells were seeded onto cell culture dishes (10 cm diameter) at a density of 5×10^4/ml. After 72 h of cultivation under standard conditions, the medium was replaced with fresh DMEM and the dishes to be exposed to hypoxia were placed into 3 airtight aluminium chambers (chamber A: 24 h; chamber B: 48 h; chamber C: 72 h) connected via a tube system to a vacuum pump and an N_2 gas cylinder. To obtain hypoxic conditions, oxygen was evacuated and displaced by N_2 (99.9%) inflow. Gas evacuation and inflow (–0.3/+1.3 bar) were stop-cock controlled and the inner chamber pressure was monitored with a barometer. In order to increase gas exchange, the chambers were placed in a shaking water bath (37°C; 45 rpm). Gas exchange was performed every 2 min for 22 min in total and resulted in final oxygen concentrations in the medium of approximately 0.5% (±1.8) as confirmed by polarographic needle electrode measurements using a pO_2 Histograph (Sigma Eppendorf, Hamburg, Germany). The hypoxic chambers A, B and C were subsequently placed in a 37°C incubator for 24, 48 or 72 h, respectively. Normoxic control cells were concurrently maintained under standard conditions. After 24, 48 and 72 h of hypoxic exposure, a subset of cells was allowed to reoxygenate for 24 h under standard conditions.

Determination of Cell Vitality and Cell Proliferation

Cells were rinsed twice with PBS and detached with trypsin/EDTA, washed with 5 ml DMEM supplemented with 10% FCS and collected in tubes (2 aliquots per sample) in order to determine intracellular esterase activity and cell membrane integrity reflecting cell vitality (n = 3). Following centrifugation (112 *g*; 3 min) the supernatant was removed, the cells resuspended in 2 ml PBS and centrifuged at 112 *g* for 3 min. One cell aliquot (2.5×10^5 cells) was fixed in 1% paraformaldehyde/PBS (30 min; room temperature, RT), washed twice with PBS and stored at 4°C until further analysis. The matching second aliquot was incubated for 30 min in a 0.9% NaCl solution containing 0.5 μM calcein AM. In viable cells, non-fluorescent membrane-permeant calcein AM is cleaved by intracellular esterases, resulting in an intense green fluorescence at an excitation wave length of 495 nm and an emission wave length of 515 nm. Cell aliquots for the analysis of the human proliferation marker Ki-67 were spun down by centrifugation (350 *g*; 5 min; 15°C) and resuspended in 250 μl of PBS/1% BSA/0.025% saponin containing monoclonal mouse antibody to human Ki-67 (clone MIB-1; 0.8 μg/ml; 1 h; RT). Subsequently, cells were washed twice with PBS/1% BSA and incubated with 250 μl of PBS/1% BSA containing AlexaFLUOR 488 goat anti-mouse IgG (2 μg/ml; 20 min; RT), washed again and resuspended in 400 μl PBS. Propidium iodide (1 μg/ml) was used as a counterstain for the calcein aliquots to mark the nuclei of dead cells and for the Ki-67 aliquots in order to determine the DNA content. Cell-associated fluorescence was determined by flow cytofluorometry employing the FACSCalibur™ flow cytofluorometer (Becton-Dickinson, Heidelberg, Germany; low-power argon laser excitation at 488 nm) and CellQuest Pro™ software. For each analysis, approximately 10,000 gated events were collected.

uPA and PAI-1 ELISA

After removal of cell culture supernatant aliquots, adherent cells were rinsed twice with ice-cold PBS and subsequently lysed in 900 μl buffer containing 0.05 M Tris/HCl, pH 8.5, 0.1 M NaCl, 10 mM EDTA, 0.5% Tween 20 and 0.1% Triton X-100. Cell lysates were rotated for 24 h at 4°C, then, as for the cell culture supernatants, spun at 15,000 *g* for 20 min at 4°C and the resulting cell extracts and cell culture supernatants stored at –20°C until further use. Protein concentration of detergent extracts was determined with the BCA (bicinchoninic acid) protein assay kit using BSA as the standard. uPA and PAI-1 antigen were measured by ELISA as described previously [23]. All measurements were performed in duplicate. uPA and PAI-1 antigen values for cell lysates as well as for supernatants are expressed as nanogram analyte/milligram protein of the cellular detergent extract.

ELISA for uPA-PAI-1 Complex Formation

Complex formation between PAI-1 and uPA was assessed by means of a '4-stage/2-site' complex ELISA according to Grebenschikov et al. [24]. Active uPA and PAI-1 fractions in the supernatants were calculated according to their stoichiometric reaction characteristics (1:1) and molecular weight (uPA: 52 kDa; PAI-1: 54 kDa) from the absolute amounts of uPA, PAI-1 and uPA-PAI-1 complex content.

Statistical Analyses

Relative changes in protein expression owing to submission to hypoxic conditions or hypoxic conditions with subsequent reoxygenation phase in relation to the 24-hour normoxic value and between hypoxic and reoxygenating conditions of 4–5 individual experiments were analysed by means of the Mann-Whitney U test for independent samples. For the analysis of overall cell numbers, the Student t test for unpaired samples of 6–8 individual experiments was used. In both tests, $p \leq 0.05$ was considered to be statistically significant. All tests were analysed using the statistical package SPSS, release 12.0.1 for Windows (SPSS Inc., Chicago, Ill., USA).

Results

Cell Vitality and Cell Proliferation as a Consequence of Hypoxic Treatment

In order to assess the influence of hypoxic exposure and subsequent reoxygenation on cell viability and proliferative capacity, the 4 cell lines VH7, BHY, FaDu and CAL27 were analysed for intracellular esterase activity and proliferation-associated Ki-67 expression. As shown in table 1, hypoxia and subsequent reoxygenation induced a significant reduction in adherent cell number ($p \leq 0.005$; $p \leq 0.001$) in all 4 cell lines tested. However, the fraction of viable adherent cells (calcein positive) did not significantly differ between cells kept under normoxic and hypoxic growth conditions and after reoxygenation. Staining for the Ki-67 antigen also revealed no significant changes between adherent cells exposed to hy-

Table 1. Cell viability and proliferative capacity

	Cell count, %			
	VH7	BHY	FaDu	CAL27
Adherent cells				
N	100 ± 7	100 ± 34	100 ± 18	100 ± 41
H	53 ± 6*	19 ± 8*	20 ± 6**	15 ± 2*
R	55 ± 7*	19 ± 6*	16 ± 6**	18 ± 3*
Calcein-positive cells				
N	91 ± 14	72 ± 22	68 ± 26	83 ± 18
H	92 ± 7	70 ± 20	82 ± 16	83 ± 12
R	94 ± 6	82 ± 13	82 ± 9	82 ± 11
Ki-67-positive cells				
N	3 ± 1	41 ± 11	62 ± 3	64 ± 7
H	5 ± 2	65 ± 25	71 ± 11	66 ± 21
R	5 ± 2	58 ± 18	83 ± 8	65 ± 21

Percentage of adherent (n = 6–8), calcein-positive and Ki-67-positive cells (n = 3) in VH7, BHY, FaDu and CAL27 cells at normoxia, and after exposure to hypoxia and hypoxia + 24-hour reoxygenation period. Data are represented as average values over all 3 time points (24, 48 and 72 h) ± SD. N = Normoxia; H = hypoxia; R = hypoxia + 24-hour reoxygenation period. * $p \leq 0.005$; ** $p \leq 0.001$ (Student t test for unpaired samples).

Table 2. Comparison of cell-associated and secreted uPA and PAI-1 protein in the cell lines VH7, BHY, FaDu and CAL27 at 24 h of normoxia

	uPA			PAI-1		
	CA	SN	R	CA	SN	R
VH7	2.4	3.4	1.4	296	21,555	73
BHY	47	432	9	332	4,099	12
FaDu	17	299	18	40	480	12
CAL27	41	687	17	10	190	19

Determination of the n-fold change in median secreted uPA and PAI-1 amounts versus median cell-associated protein content in VH7, BHY, FaDu and CAL27 cells. SN = Supernatant (ng/mg protein in cellular detergent extract); CA = cell-associated (ng/mg protein in cellular detergent extract); R = ratio (n-fold change between secreted and cell-associated protein).

poxia and subsequent reoxygenation in comparison to cells kept under normoxic conditions.

Determination of Cell-Associated and Secreted uPA Levels by Means of ELISA

The highest amounts of cell-associated uPA at 24-hour normoxia were found in the cell line BHY, followed by the cell lines CAL27, FaDu and VH7 (table 2). Significant changes in the median amounts of cell-associated uPA protein relative to the 24-hour normoxic value after exposure to hypoxia were seen in cell lines VH7, BHY ($p \leq 0.05$) and FaDu ($p \leq 0.01$). Upon reoxygenation, significant changes in cell-associated uPA protein were seen for the cell lines VH7 and BHY ($p \leq 0.05$), in FaDu ($p \leq 0.01$) and CAL27 cells ($p \leq 0.05$; $p \leq 0.01$; fig. 1).

The uPA content in the supernatant at 24-hour normoxia was highest in CAL27 cells, followed by the cell lines BHY, FaDu and VH7 (table 2). The n-fold change between secreted and cell-associated uPA was highest in FaDu cells, followed by CAL27, BHY and VH7 cells (table 2). Significant down-regulation in secreted uPA protein amounts relative to the 24-hour normoxic value during hypoxia was observed in the cell lines BHY ($p \leq 0.05$) and FaDu ($p \leq 0.01$; fig. 1). Upon reoxygenation, significant up-regulation of secreted uPA protein was observed in the cell lines VH7 ($p \leq 0.05$), BHY ($p \leq 0.05$) and CAL27 ($p \leq 0.01$).

Comparison of median cell-associated uPA protein amounts between hypoxic and reoxygenated cells furthermore revealed the statistically significant increase in uPA protein upon reoxygenation in the cell lines VH7 (24 h; 48 h) and CAL27 (24 h; 72 h). For secreted uPA protein this observation was made for BHY, CAL27 and FaDu cells exposed to 24-hour hypoxia and subsequent reoxygenation ($p \leq 0.05$; $p \leq 0.01$; fig. 1).

Determination of Cell-Associated and Secreted PAI-1 Levels by Means of ELISA

The highest cell-associated PAI-1 amounts were found in the cell line BHY, followed by the cell lines VH7, FaDu and CAL27 (table 2). Exposure to hypoxia induced a significant increase in median cell-associated PAI-1 ($p \leq 0.05$; $p \leq 0.01$) in all cell lines when compared to the 24-hour normoxic value. On reoxygenation, changes in median cell-associated PAI-1 content relative to the 24-hour normoxic value revealed a significant increase ($p \leq 0.05$; $p \leq 0.01$) after exposure to hypoxia in VH7 and CAL27 cells. This observation was also made for BHY cells up to 48 h of hypoxic exposure ($p \leq 0.05$) and in FaDu cells after 24-hour exposure to hypoxia (fig. 2).

The cell lines also varied with regard to the PAI-1 concentration in the supernatant at 24-hour normoxia (table 2). The highest amounts were found in cell line VH7, followed by the cell lines BHY, FaDu and CAL27. The n-fold change between secreted and cell-associated PAI-1 was highest in VH7 cells, followed by CAL27, BHY and

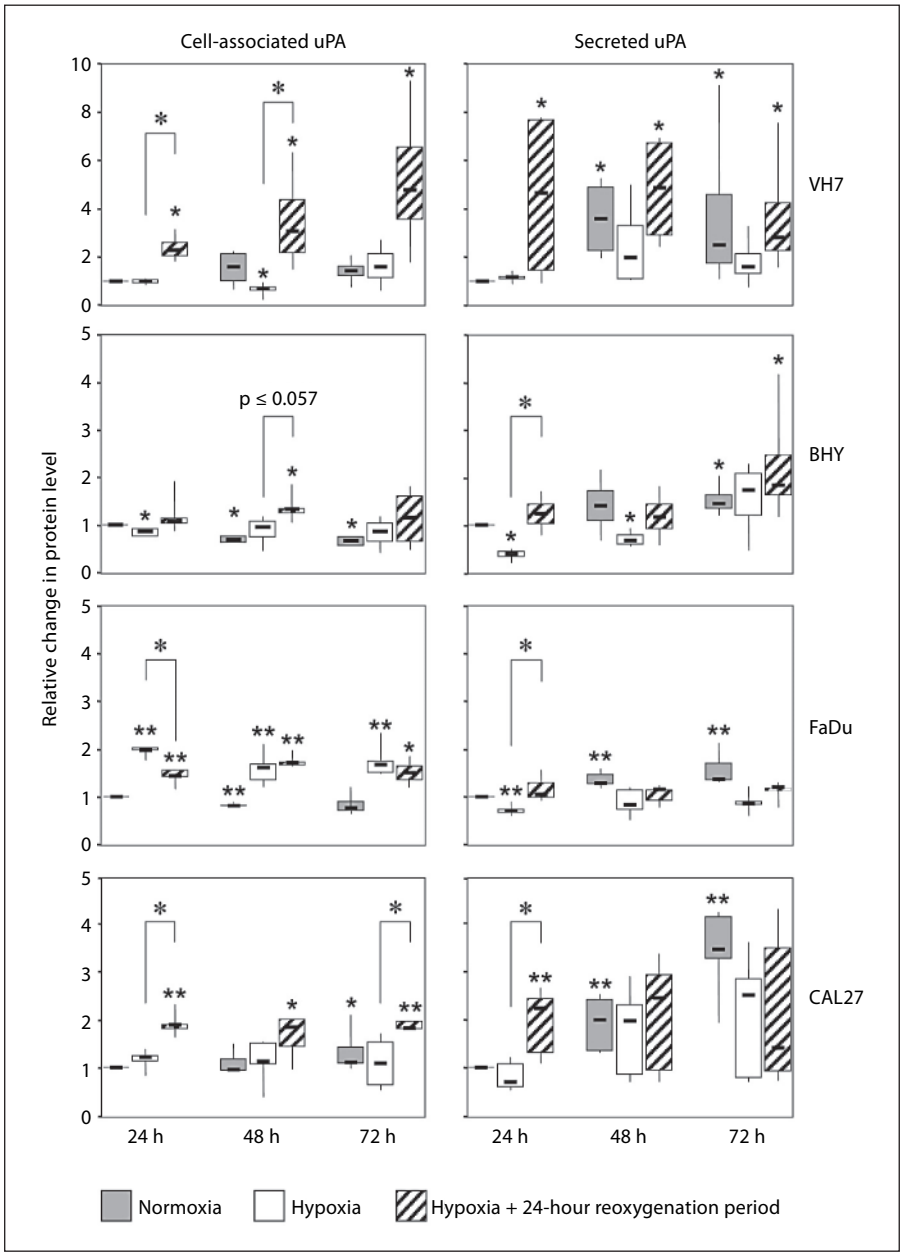

Fig. 1. Time- and oxygen-dependent changes in cell-associated (detergent extracts) and secreted uPA protein levels in the cell lines VH7, BHY, FaDu and CAL27. Values are shown as median (crossbeam), range (vertical bars) and 75 and 25% quartile (column). Changes in protein expression for hypoxia and hypoxia + 24-hour reoxygenation period were calculated relative to the 24-hour normoxic value and between hypoxic and reoxygenated cells (n = 4–5). Data were analysed by means of the Mann-Whitney U test for independent samples. * $p \leq 0.05$; ** $p \leq 0.01$.

FaDu cells (table 2). In comparison to the 24-hour normoxic value, a significant increase in secreted PAI-1 after exposure to hypoxia was observed in the cell lines BHY, FaDu ($p \leq 0.05$) and CAL27 ($p \leq 0.01$). Again, changes in the amount of secreted PAI-1 after exposure to hypoxia and subsequent reoxygenation relative to 24-hour normoxia revealed a continuous significant increase in secreted PAI-1 ($p \leq 0.05$; $p \leq 0.01$) in all cell lines with the exception of VH7 (fig. 2). When comparing the changes in secreted PAI-1 protein levels between hypoxic and reoxygenated cells, a continuous increase in all cell lines was observed. These changes were significant in BHY, FaDu and CAL27 cells ($p \leq 0.05$; $p \leq 0.01$; fig. 2).

Determination of Complex Formation between uPA and PAI-1 in the Supernatant

One set of samples from each cell line was used to assess complexes of uPA and PAI-1 present in the cell culture supernatant (table 3). uPA-PAI-1 complex formation indicates once reactive uPA and PAI-1.

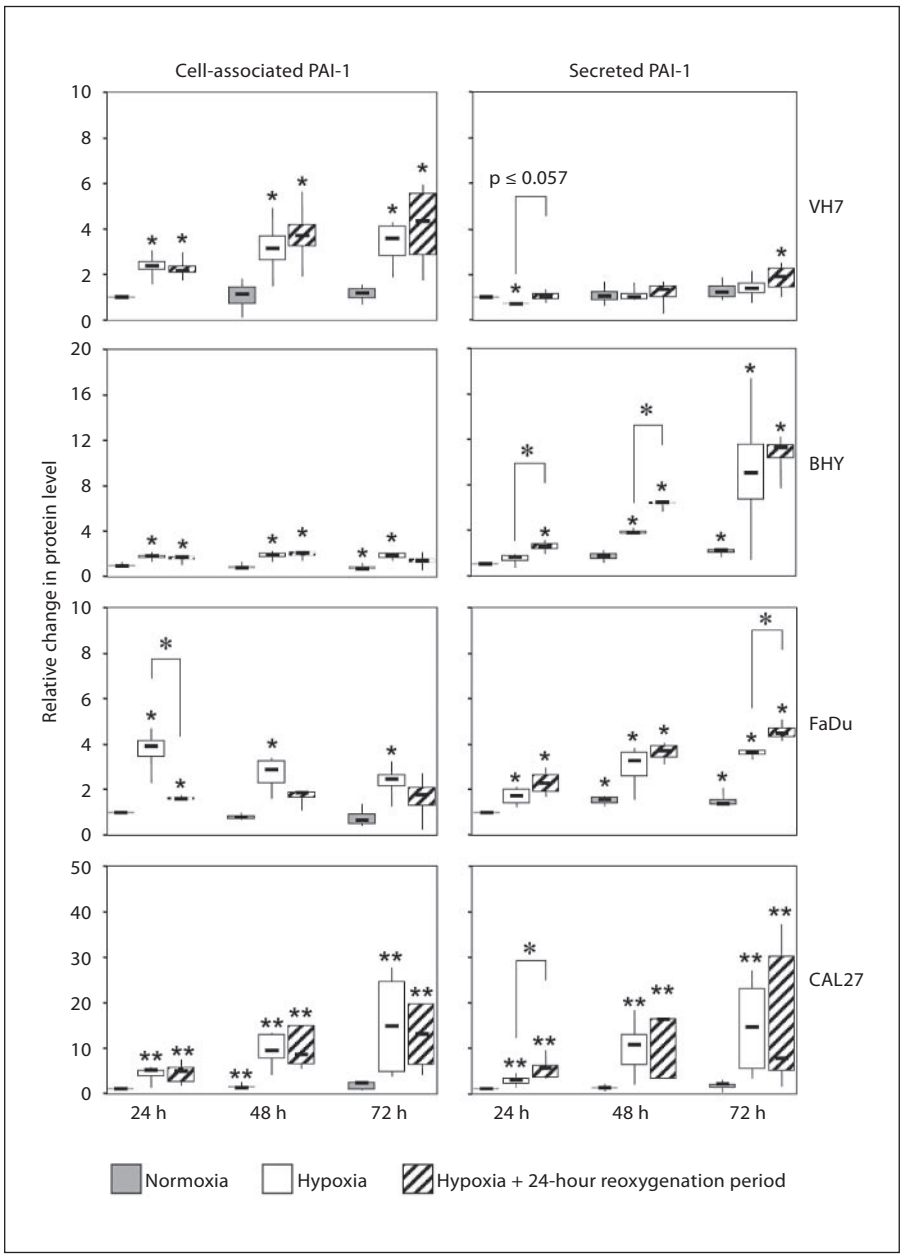

Fig. 2. Time- and oxygen-dependent changes in cell-associated (detergent extracts) and secreted PAI-1 protein levels in the cell lines VH7, BHY, FaDu and CAL27. Values are shown as median (crossbeam), range (vertical bars) and 75 and 25% quartile (column). Changes in protein expression for hypoxia and hypoxia + 24-hour reoxygenation period were calculated relative to the 24-hour normoxic value and between hypoxic and reoxygenated cells (n = 4–5). Data were analysed by means of the Mann-Whitney U test for independent samples. * $p \leq 0.05$; ** $p \leq 0.01$.

The very low amounts of secreted uPA in the supernatant of VH7 cells were nearly completely complex bound, with values ranging from 60 to 99.5%. Quantification of complex formation by BHY and FaDu cells revealed that during reoxygenation, up to 32% of the secreted uPA was complex bound in BHY cells and up to 26% in FaDu cells. Although secreted uPA amounts were highest in the supernatant of CAL27 cells, the quantity of complex-bound secreted uPA was very low regarding this cell line, with values between 1 and 10% during hypoxia and reoxygenation.

During hypoxia and reoxygenation, high levels of secreted PAI-1 were observed in the supernatants of VH7 and BHY cells. In the supernatant of hypoxic and reoxygenated VH7 cells, the amount of complex-bound PAI-1 ranged from 1 to 5%, whereas in reoxygenated BHY cells, up to 10% of the secreted PAI-1 was complex bound. Values in hypoxic and reoxygenated FaDu cells ranged from 21 to 84% and in hypoxic CAL27 cells from 9 to 72%.

Table 3. Percentage of complex-bound uPA and PAI-1 in the supernatant

Condition	VH7				BHY				FaDu				CAL27			
	uPA SN ng/ml	uPA CB %	PAI-1 SN ng/ml	PAI-1 CB %	uPA SN ng/ml	uPA CB %	PAI-1 SN ng/ml	PAI-1 CB %	uPA SN ng/ml	uPA CB %	PAI-1 SN ng/ml	PAI-1 CB %	uPA SN ng/ml	uPA CB %	PAI-1 SN ng/ml	PAI-1 CB %
N (24 h)	0.05	80	70	0.8	30	18	44	13	19	12	4	61	90	1.5	3	42
H (24 h)	0.12	99.5	43	2	5.5	30	51	3	13	17	5	42	41	1	5	9
R (24 h)	0.35	93	125	3	27	32	85.5	10	24	26	8	84	97	2.5	10	26
N (48 h)	0.1	81	113	2.7	57	23	85	16	49	21	11	98	208	2	7	73
H (48 h)	0.11	60	107	1	12	18	80	3	15	25.5	12	32.5	59	10	8	72
R (48 h)	0.11	89	142	5	31	26	94	9	15	20	10	30.5	82	3	13	19
N (72 h)	0.25	61	156	0.8	92	24	149	15	73	18	15	30	785	0.03	15	2
H (72 h)	0.1	98.5	161	1.2	19	12	111	2	14	10	10	47	65	4	17	16
R (72 h)	0.22	99	220	5	25	30	122	6	16	15	10	21	88	5	22	21

Fraction of once active uPA and active PAI-1 present in the supernatant of the cell lines VH7, BHY, FaDu and CAL27, capable of forming uPA/PAI-1 complexes at normoxia, and after exposure to hypoxia and hypoxia + 24-hour reoxygenation period. Active uPA and PAI-1 fractions in the supernatants were calculated according to their stoichiometric reaction characteristics (1:1) and molecular weight (uPA: 52 kDa; PAI-1: 54 kDa; uPA-PAI-1 complex: 106 kDa) from the absolute amounts of uPA, PAI-1 and uPA-PAI-1 complex. SN = Supernatant (total amount of secreted protein); CB = complex bound; N = normoxia; H = hypoxia; R = hypoxia + 24-hour reoxygenation period.

Discussion

The present study analysed the influence of various exposure times to prolonged hypoxia (<0.5% O_2) with and without subsequent 24-hour reoxygenation on uPA and PAI-1 protein expression in 3 human cell lines originating from SCCHN. We chose an in vitro approach to study the isolated effect of hypoxia and reoxygenation in a standardised environment thereby avoiding interfering effects caused by treatment, such as radiation or chemotherapy.

In our study, hypoxia provoked a significant reduction in cell number (approximately 80%), but resistant cells, determined by their viability (calcein-positive cells) and metabolic activity (Ki-67-positive cells), could still be detected after up to 72 h of hypoxic conditions (table 1). In this respect, we would like to mention that in vitro exposure to hypoxia is not per se toxic or growth inhibitory provided that sufficient glucose and growth-promoting factors are available [25]. Nevertheless, if only residual amounts of oxygen are present for a longer period of time, as in this experimental set-up, proliferation can pause. As a result, depending on the exposure time to hypoxia and environmental pH, cells become quiescent but remain viable and can resume cell growth upon re-establishment of more favourable environmental conditions. Accordingly, the proliferation marker Ki-67 was expressed in all of the 3 tumour cell lines even after up to 72 h of hypoxic exposure. Ki-67 is expressed during the active phases of the cell cycle (G_1, S, G_2 and M phase) but not in quiescent cells (G_0 phase) [26]. Cells expressing Ki-67 protein maintain their proliferative potential and can resume the cell cycle once the growth-arresting factors are eliminated [26]. We observed, via propidium iodide staining of the nuclei of fixed cells, the accumulation of both normoxic but also hypoxic tumour cells in the G_1 phase. Subsequent reoxygenation induced a shift towards the G_2/M phase, thus indicating that the cells are still capable of reentering the cell cycle even after prolonged exposure to severe hypoxia [Mengele and Sprague, unpubl. results]. These findings are in accordance with the findings of Krtolica and Ludlow [27], who observed cell cycle arrest in human ovarian carcinoma cells exposed to hypoxia. This arrest was reversible upon reoxygenation of the cultures. However, the proliferative ability of the hypoxic cells during reoxygenation was found to be severely impaired in comparison to normoxic cells.

We next analysed how exposure to hypoxia and subsequent reoxygenation will influence the PA system and observed that changes in cell-associated uPA levels in response to hypoxia varied between the cell lines. Significant up-regulation relative to the baseline at 24 h of nor-

moxia was observed in FaDu cells, whereas mostly steady-state uPA protein levels were seen in the other 3 cell lines. On reoxygenation, however, a significant increase in cell-associated uPA protein levels was found in VH7, BHY and CAL27 cells.

Regarding secreted uPA, our observations of hypoxia-induced down-regulation of uPA are in line with the findings of Kroon et al. [12] and Graham et al. [13]. Both groups, using different cell models, found that exposure to hypoxia (up to 72 and 24 h, respectively) resulted in decreased uPA levels secreted by human microvascular endothelial cells and by breast cancer epithelial cells. However, on reoxygenation we did observe a trend towards increased protein secretion in all cell lines indicating protein retention during hypoxia and release during reoxygenation.

We also observed a time-dependent and significant increase in cell-associated PAI-1 in all 4 cell lines examined. Moreover, although hypoxia induced significantly accelerated secretion of PAI-1 by the tumour cell lines into the culture medium, subjection to reoxygenation even further enhanced PAI-1 secretion. Regarding published data, opposing statements concerning hypoxia-induced PAI-1 protein secretion by very diverse cell lines exist. For instance, after hypoxic exposure, human proximal renal tubular cells show a significant increase in PAI-1 secretion [28], whereas in hypoxic corneal epithelial cells, decreased PAI-1 secretion is associated with augmented uPA activity [11].

In order to determine the active fraction of uPA and PAI-1 in the supernatants, the amount of complex formation between uPA and PAI-1 was assessed, since only the active forms of the uPA and PAI-1 molecules can interact with each other to form uPA-PAI-1 complexes. Exposure to hypoxia and reoxygenation did not result in the reduction of uPA-and PAI-1 activity in comparison to normoxic growth conditions, as shown by their ability to form complexes in any of the cell lines investigated. However, the differences observed between the 4 cell lines regarding the amounts of complex formation during hypoxia and reoxygenation might be indicative of the invasive behaviour of the tumour cells. With regard to the absolute amounts of both PAI-1 and uPA in the supernatants, the highest amount of complex formation was observed for the highly invasive cell line BHY, followed by the moderately invasive CAL27 and marginally invasive FaDu cell lines.

Recent in vitro and in vivo studies [29, 30] have shown that reoxygenation of hypoxic tumour cells can induce free radical formation, leading to the nuclear accumulation of hypoxia-inducible factor-1 (HIF-1), a major oxygen homeostasis regulator. Under normoxic conditions the heterodimeric transcription factor HIF-1 is rapidly degraded by the proteasome [31]. However, hypoxia stabilises its hypoxia-regulated subunit HIF-1α, thus permitting dimerisation with its oxygen-independent subunit HIF-1β to form the active HIF-1 transcription complex. This complex can then bind to hypoxia-regulatory elements in the nucleus, resulting in the transcription of genes, such as PAI-1, which are involved in tumour metabolism, cell growth and angiogenesis [6, 32]. Nuclear accumulation of HIF-1 in response to reactive oxygen during reoxygenation, as observed by Moeller et al. [30], might therefore be responsible for our observation of increased PAI-1 secretion upon reoxygenation.

A rise in cell-associated and secreted PAI-1 after exposure to hypoxia which still continues after reoxygenation could have various clinically relevant consequences. Although PAI-1 inhibits the proteolytic activity of uPA, and as such is assumed to be an inhibiting factor in malignant progression, it is actually a marker for aggressiveness of malignant tumours [33]. PAI-1 can inhibit apoptosis in transformed and non-transformed cells [34] and thus potentially increase the aggressiveness of a tumour. Tumours lacking PAI-1 display lower proliferative and higher apoptotic indices, as observed in PAI-1 gene-deleted mice [35]. On the other hand, PAI-1 regulates cell adhesion and detachment of cells from the ECM by binding to the ECM protein vitronectin, thus facilitating the dissemination of tumour cells to distant organs [36]. A third possible consequence could be changes in tumour angiogenesis, since PAI-1 is pro-angiogenic at nanomolar concentrations and anti-angiogenic at micromolar concentrations [37].

Both Nordsmark et al. [38] and Stadler et al. [39] were among the first to note that increased tumour tissue hypoxia is an indicator of poor prognosis in patients with carcinoma of the head and neck. Moreover, Stadler et al. [22] were the first to observe the direct correlation between increased tumour uPA levels and deteriorating oxygenation status. In a later study, Hundsdorfer et al. [19] reported elevated uPA and PAI-1 levels in oral cavity tumours and both uPA and PAI-1 content seem to be strong independent prognostic factors for disease-free survival in patients with head and neck tumours. Hypoxia is believed to play a critical role in the malignant progression in these tumours and the fundamental role of uPA and PAI-1 in tumour invasion and metastasis is a well-established fact [6, 40]. To our knowledge, our study is the first to show that not only hypoxia but also reoxygenation can

lead to a significant increase/accumulation of cell-associated and secreted PAI-1 and cell-associated uPA in head and neck tumour cells. Reoxygenation during radiation therapy is a welcomed side effect and some efforts have been made to increase this effect, such as breathing of carbogen during radiation or correction of haemoglobin levels [41, 42]. However, our study has shown that reoxygenation does not seem to counteract the effects induced by hypoxia on the PA system. Two different mechanisms might therefore be competing during reoxygenation: improvement in radiosensitivity and concomitant increase in malignancy. The ultimate aim in radiation therapy is to kill tumour cells and spare surrounding healthy tissue; however, even if a tumour cell is doomed to die within the next few division cycles after exposure to radiation, the time left is possibly still enough to produce sufficient amounts of 'harmful' protein and thus initiate a path for invasion and metastasis for surviving tumour cells.

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