Effects of hypoxia and reoxygenation on the expression levels of the urokinase-type plasminogen activator, its inhibitor plasminogen activator inhibitor type-1 and the urokinase-type plasminogen activator receptor in human head and neck tumour cells

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Abstract. One aim during oncological radiation therapy is to induce reoxygenation in hypoxic tumours in order to enhance radiosensitivity and ultimately increase cell death. In squamous cell carcinomas of the head and neck (SCCHN), hypoxia is considered a pivotal physiological modulator for malignant progression, whereby the plasminogen activation system is involved in overlapping functions such as the shaping of the extracellular matrix, cell proliferation and signal transduction. Since little is known about reoxygenation and the plasminogen activation system in SCCHN, three human SCCHN cell lines (BHY, FaDu, and CAL27) and a non-transformed control cell line (VH7) were exposed to hypoxic (<0.5% O_2) conditions for up to 72 h and subsequently reoxygenated for 24 h at normoxic conditions. The mRNA expression of the urokinase-type plasminogen activator (uPA), the plasminogen activator inhibitor type-1 (PAI-1) and the urokinase-type plasminogen activator receptor (uPAR) was assessed by means of real-time semi-quantitative RT-PCR, and the protein expression was determined by immunoenzymometric quantification (ELISA). Both hypoxia and reoxygenation induced statistically significant changes in uPA, PAI-1 and uPAR mRNA and protein levels in the various cell lines investigated, showing that oxygen tension is a strong modulator of the plasminogen activation system *in vitro*. However, no uniform correlation pattern was found between the mRNA and protein levels analysed over all three time-points (24, 48, and 72 h) and oxygen treatment variants (N, H, R) nor according to oxygen treatment conditions over all three time-points. Changes in oxygen tension could therefore be modulating the fragile balance between the various components of the plasminogen activation system in SSCHN ultimately leading to an increased tumour matrix disruption, alterations in cell invasiveness, and the dissemination of tumour cells to distant organs.

Introduction

Tumour hypoxia is considered to be a fundamental physiological modulator of malignant progression since hypoxic tumours tend to be biologically more aggressive, resistant to radiation therapy and are therefore more likely to recur locally or metastasise (1,2). Hence, one of the aims during radiation therapy is to induce the reoxygenation of hypoxic regions within the tumour in order to increase radiosensitivity and ultimately obtain a higher cell kill (3). In squamous cell carcinomas of the head and neck (SCCHN), reoxygenation during radiation therapy has been observed. However, to date, the clinical impact of reoxygenation during therapy is not entirely clear and conflicting results regarding the correlation between reoxygenation and treatment outcome have been reported (4).

The plasminogen activation system encompasses a broad spectrum of proteolytic factors involved in physiological as well as pathological processes such as fibrinolysis, tissue remodelling and tumour invasion (5). Within this system, a gene influenced by hypoxia is that of the plasminogen activator inhibitor type-1 (PAI-1) (6), a major inhibitor of the serine protease urokinase-type plasminogen activator (uPA). Upon secretion, the short-lived inhibitory activity of PAI-1 is stabilised by binding to the extracellular matrix protein, vitronectin, a cell adhesion glycoprotein involved in tissue turnover and repair. uPA is synthesized and secreted by various

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normal and tumour cells as a nearly inactive proenzyme (pro-uPA). Via cleavage by proteases, e.g. plasmin, its proteolytic activity increases and this enzymatically highly active form of uPA (HMW-uPA) is transferred to an even higher state of activity after binding to the specific membrane-associated receptor [urokinase-type plasminogen activator receptor (uPAR)] (CD87). This HMW-uPA then converts the plasma proenzyme plasminogen to plasmin, which can either directly degrade extracellular matrix components or act indirectly by activating pro-matrix metalloproteinases (MMPs), thus facilitating cell spread. The inhibitor PAI-1 interferes with that process by interacting with and thereby inactivating the cell membrane-associated uPAR-uPA complex. As a result, the newly formed uPAR/-uPA/-PAI-1 complex is internalised by the cell, thereby initiating signal transduction and cell proliferation (7). Thus, PAI-1 not only functions as a fibrinolytic inhibitor (8) but also plays an important role in signal transduction, cell adherence and cell migration (9), both under normal and malignant conditions, including tumour invasion and metastasis (10).

Hypoxia (the state of low oxygen supply), which is a potent inducer of angiogenesis, also promotes tumour growth, invasion and metastasis. Within this context, the arising migration of endothelial cells necessitates the proteolysis of the extracellular matrix, which is achieved by significantly up-regulating uPA and uPAR expression. However, the response of the members of the plasminogen activation system to reduced oxygen availability is not uniform. For instance, the *ex vivo* cultivation of human trophoblasts, human liver-derived cells or bovine endothelial cells under low oxygen induces the upregulation of PAI-1 mRNA (6,11,12) whereas decreased PAI-1 mRNA levels are a feature of human corneal epithelial cells kept under hypoxic conditions (13). Likewise, with regard to uPA, Ito *et al* (14) observed elevated mRNA levels in the ischemic cerebral cortex of rats, while decreased uPA mRNA levels were seen in hypoxic murine lung tissue (15) and steady state uPA mRNA levels in hypoxic human microvascular endothelial cells (16). For uPAR mRNA expression after exposure to hypoxia, however, concordant results were reported: Hypoxia induces a significant upregulation of uPAR mRNA in human melanoma cells (17,18), in human vascular endothelial cells (16) and in human breast carcinoma cells (19,20).

A highly significant correlation of uPA, PAI-1 and uPAR expression levels with poor clinical outcome has been demonstrated by analysing tumour tissues obtained from patients afflicted with different types of solid malignant tumours, including those of the breast, gastrointestinal and genitourological tract, the lung, liver and brain as well as those of the oral cavity (21-25). Within this scenario, hypoxia and the proteolytic factors uPA, PAI-1 and uPAR are known to influence disease outcome negatively, as the impact of reoxygenation during cancer progression is still not clear.

The objective of this study was to elucidate the effects of hypoxia and reoxygenation on uPA, PAI-1 and uPAR mRNA and protein expression in a standardised *in vitro* environment. We therefore exposed cell lines deriving from SCCHN to hypoxia and subsequent reoxygenation and assessed the mRNA expression of uPA, PAI-1 and uPAR by means of real time semi-quantitative RT-PCR and the protein expression with immunoenzymometric quantification (ELISA).

Materials and methods

Cultivation of cells under normoxic and hypoxic conditions. The human adherently growing head and neck tumour cell lines BHY (ACC 404) and CAL27 (ACC 446) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The head and neck tumour cell line, FaDu (ATCC HTB-43), and the human foreskin cell line, VH-7, were kindly provided by M. Baumann (Dresden) and P. Boucamp (Heidelberg), respectively. Plastic ware for cell cultivation was obtained from TPP (Biochrom AG, Berlin, Germany). The cells were seeded onto cell culture dishes (10 cm Ø) at a density of $5x10^4$/ml. All cell lines were cultivated in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C in Dulbecco's modified Eagle's medium (DMEM; 4500 mg/l D-Glucose, 25 mM HEPES, w/o sodium pyruvate; Invitrogen, Karlsruhe, Germany) supplemented with 10% foetal calf serum (Biochrom AG). The cells from passages 10 to 25 were used for the experiments. After 72-h cultivation under standard normoxic conditions, the medium was replaced with fresh DMEM and the dishes to be exposed to hypoxia were placed into 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. In order 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 ~0.33% (±0.18) as confirmed by polarographic needle electrode measurements using a pO_2 histograph (Sigma-Eppendorf pO_2 Histograph, Hamburg, Germany). The hypoxic chambers A, B and C were subsequently placed in a 37˚C incubator for 24 h (short), 48 h (intermediate) or 72 h (long), respectively. Normoxic control cells were concurrently maintained under standard conditions. After 24-, 48- and 72-h hypoxic exposure, a subset of cells was allowed to reoxygenate for 24 h under standard conditions.

Isolation of mRNA from cell lines and cDNA synthesis. Total RNA of adherently growing cells was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and subsequently transcribed (TaqMan Reverse Transcription Reagents; Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany), both according to the manufacturer's instructions. One reverse transcription reaction contained 3.5 μl total RNA in RNase-free water, 0.25 μl Multiscribe Reverse Transcriptase (50 U/μl), and 0.2 μl RNase inhibitor (20 U/μl) to which 1 μl 10X RT-buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 2.2 μl 25 mM $MgCl_2$, 2 μl dNTP mixture (2.5 mM each dATP, dCTP, dGTP, dTTP) and 0.5 μl random hexamers (50 μM), were added. Subsequently, the reaction mixtures were incubated for 10 min at 25˚C to anneal the hexanucleotides. cDNA synthesis was performed

Table I. List of used primers and probes for real time RT-PCR.

Gene (human)	Oligonucleotide primer and probe sequence 5'--3'	Fragment size (bp)	GenBank accession no.
uPA	F 5'-gcttgctcaccacaacgaca-3' R 5'-ttcagctgctccggatagagata-3' T 5'-FAM-aagatccgttccaaggagggcaggt-TAMRA-3'	192	NM_002658
PAI-1	F 5'-ctcctggttctgcccaagtt-3' R 5'-tcgtgaagtcagcctgaaac-3' T 5'-FAM-aagtcgacctcaggaagcccctagagaga-TAMRA-3'	109	NM_000602
uPAR	F 5'-agaatggccgccagtgtta-3' R 5'-ggttgcacagcctcttaccata-3' T 5'- FAM-ctttggtttttcggttcgtgagtgcc-TAMRA-3'	164	NM_002659
28S rRNA	F 5'-ttgaaaatccgggggagag-3' R 5'-acattgttccaacatgccag-3' T 5'-FAM-tcaccttgggagacctgctgcggat-TAMRA-3'	99	M11167

uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor type-1; uPAR, urokinase-type plasminogen activator receptor.

for 30 min at 48˚C, followed by an enzyme inactivation step for 5 min at 95˚C. cDNA was stored at -20˚C until further use.

Real time semi-quantitative RT-PCR (qRT-PCR). Primers and probes were designed based on alignment studies using the BLAST program and sequence database of the National Centre for Biotechnology Information. The nucleotide sequences of the primers and probes used for the analysis of uPA, PAI-1, uPAR and 28S rRNA with their corresponding accession numbers are listed in Table I. Primers and probes were designed in cooperation with and obtained from TIB MOLBIOL (Berlin, Germany). For the determination of relative expression levels, 5' nuclease real-time PCR were run on the ABI Prism 7000™ sequence detection system (Applied Biosystems, Foster City, CA, USA). The 25 μl reaction mixture consisted of 12.5 μl 2X TaqMan™ Universal Master Mix (Applied Biosystems, Germany) containing dUNTPs, MgCl$_2$, reaction buffer and AmpliTaq Gold DNA polymerase, 0.5 μl of each primer (10 pmol μl^{-1}) and 0.5 μl of the TaqManTM probe (10 pmol μl^{-1}). Cycling was started with a 10 min denaturation step at 95˚C followed by 45 cycles with 15 sec denaturation at 95˚C, and 1 min for annealing at 60˚C. For each examined gene, 4 to 5 individual experiments were carried out per cell line. Each reaction was done in triplicate.

Using these quantitative methods requires that the PCR efficiencies of all the genes are similar and ≥95%. Efficiency was determined by means of a standard curve for each gene generated by serial dilutions of the cDNA. For this purpose, the initial cDNA concentration of 100 ng/μl was serially diluted 10-fold for the real-time PCR assay according to the standard protocol from Applied Biosystems (Fig. 1). The PCR efficiency (E) was calculated by the formula: E=10$^{(1/-slope)}$ - 1, and ranged from 96-99% for the different

assays. A slope of -3.3 (±0.1) is equivalent to 100% PCR efficiency.

PAI-1, uPA, and uPAR ELISA. uPA, PAI-1 and uPAR proteins associated with and secreted by the cell lines were measured by ELISA (uPA, Imubind No. 894; PAI-1, Imubind No. 821; uPAR, Imubind No. 893; American Diagnostica Inc., Stamford, CT, USA) as described previously (26). Briefly, after the removal of the cell supernatant aliquots, the cells were rinsed twice with ice-cold PBS and 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 (all chemicals from Sigma-Aldrich, Taufkirchen, Germany). Cell lysates were rotated for 24 h at 4˚C, spun at 15,000 x *g* for 20 min at 4˚C and the resulting cell extracts were stored at -20˚C until further use. The protein concentration of the detergent extracts was determined with the BCA protein assay kit (Pierce, Perbio Science, Bonn, Germany) using bovine serum albumin as the standard. All measurements were performed in duplicate. uPA, PAI-1 and uPAR protein values contained in the cell lysates and the culture supernatants are expressed as ng analyte/mg protein in the cellular detergent extract.

Analysis of gene expression using the 2$^{-\Delta\Delta CT}$ method. Data analyses were performed with the ABI PRISM™ sequence detection software package (version 2.1; Applied Biosystems, USA). The mean target gene mRNA expression level for the three mRNA measurements was determined and the 2$^{-\Delta\Delta CT}$ method was used to calculate the relative changes in gene expression (27). For the samples exposed to hypoxia (H) and to the subsequent 24-h reoxygenation period (R), the evaluation of 2$^{-\Delta\Delta CT}$ shows the fold changes in gene expression relative to the untreated control (24-h N), i.e. $\Delta\Delta C_T$ = {[C_T (sample) - C_T 28S rRNA (sample)] - [C_T (calibrator) - C_T 28S rRNA
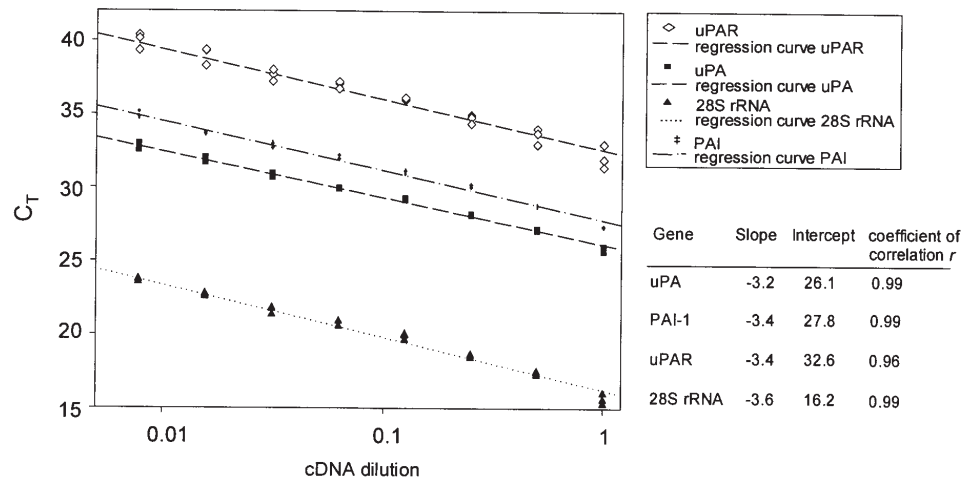
Figure 1. Regression curves for 28S rRNA, the urokinase-type plasminogen activator, plasminogen activator inhibitor type-1 and the urokinase-type plasminogen activator receptor. The initial cDNA concentration of 100 ng/μl was serially diluted 10-fold for the real-time RT-PCR assay according to the standard protocol from Applied Biosystems. The PCR efficiency (E) was calculated by the formula: $E=10^{(1/-slope)} - 1$, and ranged from 96-99% for the different assays. A slope of -3.3 ($\pm$0.1) is equivalent to 100% PCR efficiency.

(calibrator)]}. The results of the real time PCR are shown as C_T values, where C_T is defined as the threshold cycle number at which the amplified product is first detected. For the untreated control samples, $\Delta\Delta C_T$ equals 0 and 2^0 equals 1. An increased mRNA expression was defined as n-fold ≥ 2 and a decreased expression was n-fold ≤ 0.5 relative to the 24-h N expression level.

Statistical analyses. Changes in mRNA and protein expression relative to the 24-h normoxic value and between the samples exposed to hypoxia and hypoxia + reoxygenation of four to five individual experiments were analysed by means of the two-sided t-test. A p-value ≤ 0.05 was considered to be statistically significant. Levels of significance between relative uPA, PAI-1 and uPAR protein levels and relative uPA, PAI-1 and uPAR mRNA expression were calculated by the bivariate Pearson correlation. A p-value ≤ 0.05 (two-tailed) was considered statistically significant. All the tests were analysed using the statistical package SPSS Release12.0.1 for Windows (SPSS Inc., Chicago, Il., USA).

Results

Previous studies indicate that the proteolytic factors, uPA, PAI-1 and uPAR, in concert with tumour hypoxia, are key parameters influencing tumour invasion and metastasis. Little is known, however, about the impact of hypoxia and the subsequent reoxygenation on the expression of uPA, PAI-1 and uPAR in SCCHN. Therefore, the aim of the present study was to investigate the effects of short-, intermediate and long-term (up to 72 h) hypoxia and subsequent reoxygenation on the mRNA and protein expression of the proteolytic factors, uPA, PAI-1 and uPAR, in head and neck tumour cell lines in comparison to a fibroblast control cell line.

Specific amplification and correlation of RNA concentration and C_T values. Agarose gel electrophoresis (data not shown) revealed that each primer pair (Table I) amplified single bands of the predicted size for 28S rRNA (99 bp), uPA

(192 bp), PAI-1 (109 bp) and uPAR (164 bp). C_T values and the log of RNA concentrations displayed a reverse linear correlation ($R^2=0.992185$ for 28S; $R^2=0.99077$ for PAI-1; $R^2=0.990377$ for uPA; $R^2=0.959814$ for uPAR) as predicted (Fig. 1).

Relative expression of uPA mRNA in the head and neck tumour cell lines, BHY, CAL27 and FaDu, and the fibroblast control cell line, VH7, exposed to hypoxia and subsequent reoxygenation. Cultivation of the tumour cell lines under normoxic conditions for up to 72 h lead to a significant increase in uPA mRNA levels compared to the 24-h base value (Fig. 2). Cells maintained in parallel under hypoxic conditions reacted differently regarding uPA mRNA expression, which also depended on the length of exposure. After 24 h, a slight increase in uPA mRNA was observed in the FaDu and BHY cells whereas for CAL27 and the VH7 control cells a significant decline in mRNA expression was noted (Fig. 2). Reoxygenation of the once hypoxic cells for another 24 h lead to a major elevation in uPA mRNA expression in the VH7, BHY and CAL27 cells, as compared to their normoxic or hypoxic counterparts. In the cell lines, VH7, FaDu and CAL27, exposure to intermediate lengths of hypoxia (48 h) resulted in a significant reduction in uPA mRNA levels relative to the 24- and 48-h normoxic control. The subsequent 24-h reoxygenation period led to an increased uPA mRNA expression in all the cell lines. However, this was only significant for the cell lines, VH7 and BHY, in comparison to the 24-h normoxic control. Likewise, cells which were maintained under hypoxic conditions for up to 72 h followed by a 24-h reoxygenation period also resumed uPA mRNA expression although the level of expression in the tumour cell lines was considerably reduced compared to the normoxic 72-h controls.

Relative expression of PAI-1 mRNA in the head and neck tumour cell lines BHY, CAL27 and FaDu, and the fibroblast control cell line VH7, exposed to hypoxia and subsequent reoxygenation. Cultivation of the cell lines FaDu and CAL27
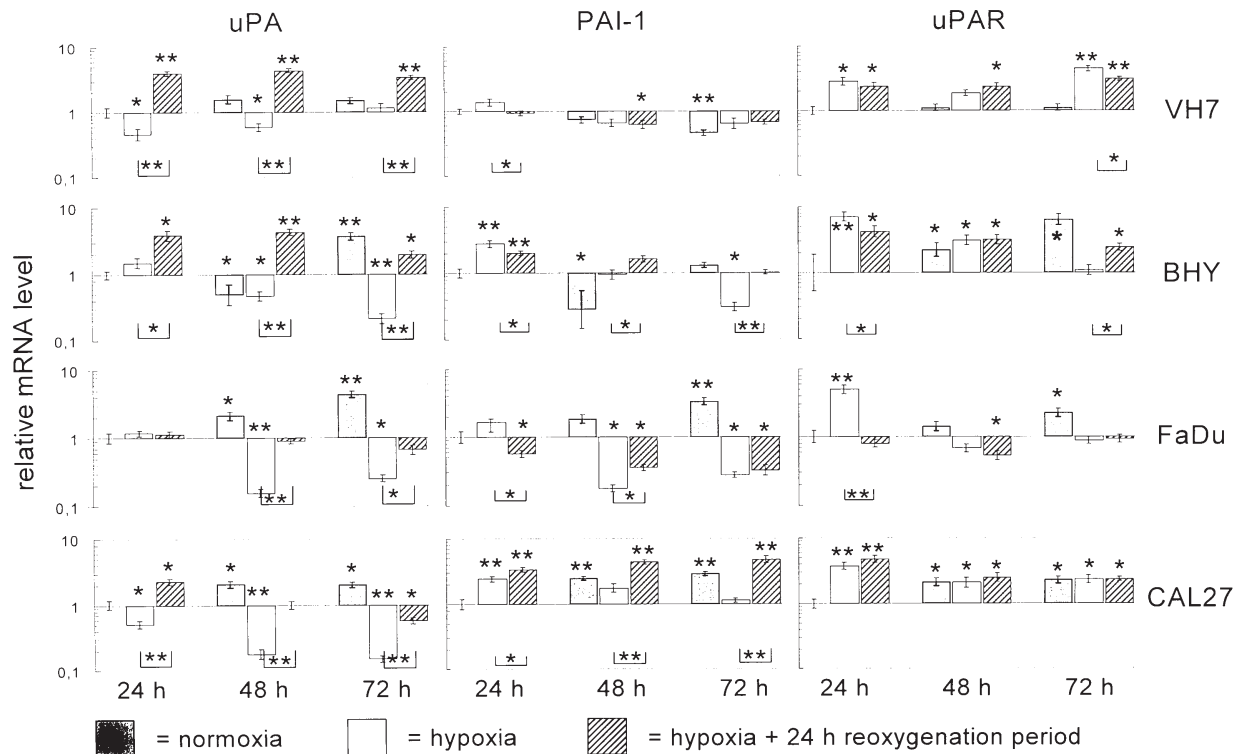
Figure 2. Time and oxygen-dependent changes of the urokinase-type plasminogen activator, plasminogen activator inhibitor type-1 and the urokinase-type plasminogen activator receptor mRNA levels for the cell lines, VH7, BHY, FaDu and CAL27. The changes in mRNA expression relative to the 24-h normoxic value and between the samples exposed to hypoxia and hypoxia + reoxygenation (|__|) of four to five individual experiments were analysed by means of the two-sided t-test. A p-value ≤0.05 was considered to be statistically significant (*p≤0.05; **p≤0.01). Error bars indicate a 95% confidence interval.

under normoxic conditions for up to 72 h lead to a significant increase in PAI-1 mRNA levels compared to the 24-h base value. The VH7 control cells behaved differently; compared to the normoxic conditions, the PAI-1 mRNA expression significantly declined at the 72-h time-point (Fig. 2). Cells maintained in parallel under hypoxic conditions responded with an increased PAI-1 mRNA expression at the 24-h time-point. Intermediate exposure to 48-h hypoxia resulted in decreased PAI-1 mRNA levels compared to the 48-h normoxic control in the cell lines VH7, FaDu and CAL27. Prolonged hypoxic exposure for up to 72 h, however, induced a considerable drop in PAI-1 mRNA expression in all the cell lines. Similar to the uPA mRNA expression, upon a 24-h reoxygenation period, PAI-1 mRNA levels changed compared to the normoxic or hypoxic mRNA expression values, being low for the tumour cell line, FaDu, and the control cell line, VH7, progressively decreasing towards base line levels in the cell line BHY, and increasing in the CAL27 tumour cell line.

Relative expression of uPAR mRNA in the head and neck tumour cell lines, BHY, CAL27 and FaDu, and the fibroblast control cell line, VH7, exposed to hypoxia and subsequent reoxygenation. Cultivation of the four cell lines under normoxic conditions for up to 72 h lead to a moderate (VH7) or significant (BHY, FaDu, CAL27) increase in uPAR mRNA levels compared to the 24-h base-value. In consequence to the 24-h hypoxic exposure, uPAR mRNA levels markedly increased in the four cell lines. An increase relative to the normoxic control was still seen at 48-h hypoxic exposure in the cell lines, VH7, BHY and CAL27. This was different

after 72 h of hypoxia. Whereas the control cell line, VH7, and the tumour cell line, CAL27, still expressed uPAR mRNA at high to moderate levels, a considerable drop in uPAR mRNA expression was observed for the BHY and FaDu tumour cell lines. Upon 24-, 48- and 72-h hypoxia with a subsequent reoxygenation period, the uPAR mRNA expression, with the exception of the FaDu cells, resumed and stayed above the normoxic control levels (24 and 48 h) even at the extended exposure of 72 h.

uPA, PAI-1 and uPAR protein content in BHY, FaDu, CAL27 and VH7 cells determined by ELISA. Cells were cultivated (see Materials and methods), the cell culture supernatants collected at the 24-, 48- and 72-h time-points and the adherent cells detached and harvested. Cells were lysed (see Materials and methods) and the uPA, PAI-1 and uPAR content was determined in the cell lysates and the cell culture supernatants. The data for the cell lysates and supernatants were pooled and plotted as the relative amounts determined for the 24-, 48- and 72-h time-points whereby the uPA, PAI-1 and uPAR protein expression levels under the 24-h normoxic conditions were set to 1 (Fig. 3).

The cell lines expressed different absolute amounts of uPA, PAI-1 and uPAR. With regard to the median total uPA concentration (cell lysate plus the supernatant related to the total protein content in the cellular detergent extract; 4 to 5 experiments, each measured in duplicate) at 24-h normoxia, the highest level of expression was determined in the CAL27 cells (720 ng/mg; range 630-1,130), followed by the BHY (472 ng/mg; range 387-472) and FaDu cells (314 ng/mg;
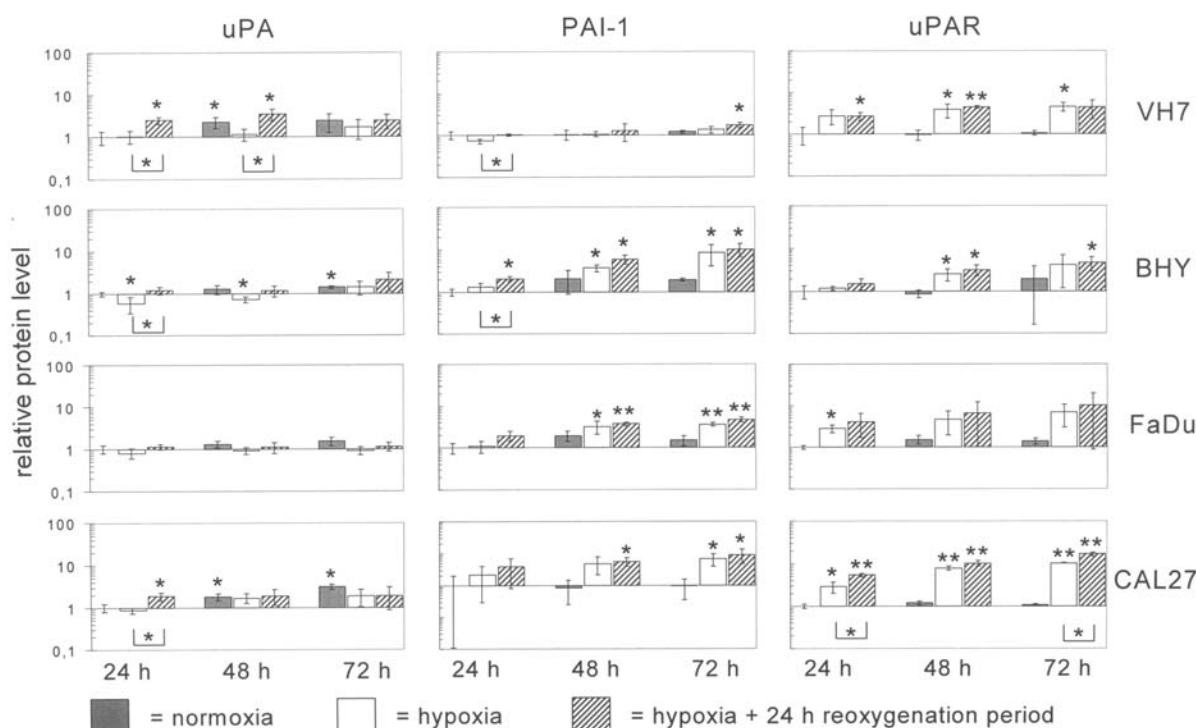
Figure 3. Time and oxygen-dependent changes of cell-associated plus secreted protein levels of the urokinase-type plasminogen activator, plasminogen activator inhibitor type-1 and the urokinase-type plasminogen activator receptor for the cell lines, VH7, BHY, FaDu and CAL27. Changes in protein expression relative to the 24-h normoxic value and between the samples exposed to hypoxia and hypoxia + reoxygenation (|__|) of four to five individual experiments, (uPAR, n=3), were analysed by means of the two-sided t-test. A p-value ≤0.05 was considered to be statistically significant (*p≤0.05; **p≤0.01). Error bars indicate standard deviation.

Table II. Correlation between mRNA and protein levels (cell-associated + secreted) of uPA, PAI-1 and uPAR in the head and neck tumour cell lines, BHY, FaDu, CAL27, and the control fibroblast cell line, VH7.

Cell line	uPA	PAI-1	uPAR
VH7	r=0.758[b] p=0.000	r=0.369 p=0.058	r=0.774[b] p=0.000
BHY	r=0.194 n.s.	r=-0.289 n.s.	r=-0.142 n.s.
FaDu	r=0.698[b] p=0.000	r=-0.556[b] p=0.003	r=-0.204 n.s.
CAL27	r=0.403[a] p=0.037	r=0.448[a] p=0.019	r=0.071 n.s.

uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor type-1; uPAR, urokinase-type plasminogen activator receptor. Pearson linear correlation analysis (two-sided) over all three time-points (24, 48, and 72 h) and oxygen treatment variants (N, H, R) between relative uPA, PAI-1 and uPAR mRNA levels and relative uPA, PAI-1 and uPAR protein levels (cell-associated + secreted) was performed for the head and neck tumour cell lines and the fibroblast control cell line ([a]p≤0.05; [b]p≤0.01) (n.s., no significance).

range 240-455) with the lowest values in the fibroblast control VH7 cells (6 ng/mg; range 4-8). The highest median total PAI-1 levels at 24-h normoxia were found in the fibroblast control VH7 cells (21,836 ng/mg; range 14,974-25,152), followed by the BHY cells (4,500 ng/mg; range 3,909-6,374), the CAL27 (624 ng/mg; range 199-1,922) and FaDu cells (563 ng/mg; range 249-647). The median total uPAR content at 24-h normoxia was highest in the BHY cells (36 ng/mg; range 23-59), followed by the VH7 control cells (8 ng/mg; range 8-18), the CAL27 (7 ng/mg; range 7-8) and FaDu cells (3 ng/mg; range 3-20). Throughout the normoxic incubation period protein values remained at the same level or increased/ decreased slightly (Fig. 3).

Although ongoing exposure to hypoxia induced the significant downregulation of uPA mRNA in all the cell lines, protein levels, with the exception of the BHY cells, did not significantly alter but remained at steady state levels. On reoxygenation a tendency towards resuming protein production could be seen in the cell lines, VH7, BHY and CAL27. PAI-1 and uPAR protein expression levels, in contrast to the mRNA expression levels, generally increased under hypoxia, being the highest at 72-h exposure indicating the continuous production of the proteolytic factors over time, even under hypoxic conditions. This level of production was not affected by a subsequent 24-h reoxygenation period.

Correlation between mRNA and protein expression. We also calculated by means of Pearson's linear correlation co-efficient (*r*) if the observed changes in mRNA expression for uPA, PAI-1 and uPAR over all three time points (24, 48 and 72 h) and all three oxygen treatment conditions (N, H, R) would correlate with the changes for total (cell-associated plus secreted) uPA, PAI-1 and uPAR protein expression (Table II). Between the uPA mRNA and protein expression,

Table III. Correlation between mRNA and protein levels (cell-associated + secreted) of uPA, PAI-1 and uPAR in the head and neck tumour cell lines, BHY, FaDu, CAL27, and the control fibroblast cell line, VH7.

Cell line	uPA			PAI-1			uPAR		
	N	H	R	N	H	R	N	H	R
VH7	r=0.907[b] p=0.001	r=0.907[a] p=0.016	r=0.907[b] p=0.007	r=-0.200 n.s.	r=-0.643 p=0.067	r=-0.151 n.s.	r=0.866[b] p=0.003	r=0.520 n.s.	r=0.546 n.s.
BHY	r=0.498 n.s.	r=-0.522 n.s.	r=-0.555 n.s.	r=-0.010 n.s.	r=-0.697[a] p=0.037	r=-0.755[a] p=0.019	r=0.694[a] p=0.038	r=-0.555 n.s.	r=-0.459 n.s.
FaDu	r=0.748[a] p=0.012	r=-0.180 n.s.	r=0.361 n.s.	r=0.429 n.s.	r=-0.799[b] p=0.010	r=-0.695[a] p=0.038	r=0.566 n.s.	r=-0.434 n.s.	r=0.523 n.s.
CAL27	r=0.733[a] p=0.015	r=-0.602 n.s.	r=0.093 n.s.	r=0.104 n.s.	r=-0.434 n.s.	r=0.879[b] p=0.002	r=0.678[a] p=0.045	r=-0.727[b] p=0.027	r=-0.644 p=0.061

N, normoxia; H, hypoxia; R, reoxygenation; uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor type-1; uPAR, urokinase-type plasminogen activator receptor. Pearson linear correlation analysis (two-sided) between relative uPA, PAI-1 and uPAR mRNA levels and relative uPA, PAI-1 and uPAR protein levels (cell-associated + secreted) according to oxygen treatment conditions over all three time-points was performed for the head and neck tumour cell lines and the fibroblast control cell line ([a]$p \leq 0.05$; [b]$p \leq 0.01$) (n.s., no significance).

the cell lines, VH7, FaDu and CAL27, showed a statistically strong, moderate and weak significant positive correlation, respectively. For PAI-1, correlation analysis revealed statistically relevant correlations for the tumour cell lines, FaDu (moderate, negative correlation) and CAL27 (weak, positive correlation). With regard to uPAR, only the control cell line, VH7, displayed a statistically significant (strong, positive) correlation between uPAR mRNA and protein levels. We next compared the correlation between changes in protein and mRNA expression according to oxygen treatment conditions over all three time points (Table III). The three cell lines, VH7, FaDu and CAL27, showed a statistically significant positive correlation between normoxic uPA mRNA and protein expression. Under hypoxic and reoxygenating conditions, only the control cell line, VH7, displayed a high, statistically significant, positive correlation between the uPA mRNA and protein. For PAI-1, correlation analysis revealed a statistically significant negative correlation for the cell lines, BHY and FaDu, under hypoxic and reoxygenating conditions, and a high, statistically significant positive correlation for the cell line, CAL27, under reoxygenating conditions. No correlation was seen under normoxic conditions. With regard to uPAR, under normoxic conditions the cell lines, VH7, BHY and CAL27, showed a statistically significant positive correlation between the uPAR mRNA and protein. Under hypoxic conditions, only the cell line, CAL27, displayed a statistically significant negative correlation. No statistically significant correlations for uPAR were observed for any cell line under reoxygenating conditions.

Discussion

Many studies using different cell and animal tumour models indicate that tumour hypoxia is a key parameter influencing malignant progression in head and neck cancer. However, little is known about the impact of reoxygenation in SCCHN under therapy (4). Using a real-time semiquantitative RT-PCR method, we analysed the influence of hypoxia (<0.5% O_2, up to 72 h) and the subsequent 24-h reoxygenation period on three components of the plasminogen activation system (uPA, PAI-1, uPAR), in three different cell lines derived from human SCCHN and a fibroblast control cell line . These three components are known to be associated with poor outcome in patients afflicted with various types of cancer (21,24,25).

In all cell lines examined, we saw significant oxygen- and time-dependent changes in uPA, PAI-1 and uPAR mRNA levels. Regarding the uPA mRNA expression, our findings are in agreement with data described in the literature showing that hypoxia leads to a reduction in uPA mRNA expression. For instance, it has been reported that in murine lung tissue, uPA mRNA expression decreases significantly after hypoxic exposure (15), or remains at steady state levels in human vascular endothelial cells (16). In our cell lines tested, reoxygenation triggered the upregulation of the uPA mRNA expression after hypoxia. To our knowledge, no study has yet described the effects of reoxygenation on uPA mRNA synthesis *in vitro*. However, indirect evidence is given in a study by Mahabeleshwar and Kundu (28) revealing that the reoxygenation of human breast cancer cells can stimulate NF-κB-dependent uPA promoter activity and ultimately lead to increased uPA synthesis and secretion.

With regard to changes in the PAI-1 mRNA levels in our cell lines tested under hypoxia, the most significant rise occurred after 24-h exposure to hypoxia which is in accordance with published data (11,12,15,30). Remarkably, the increase in PAI-1 mRNA levels seen in these earlier studies was not affected by the oxygen concentration during hypoxic exposure,

which in these studies ranged from 1 to 8%. Furthermore, all of these studies only tested hypoxic periods ranging from 2 to 24 h. In our study, however, a longer time of exposure of up to 72 h under hypoxic conditions of below 0.5% oxygen, compared to the 24-h data, resulted in a considerable drop in PAI-1 mRNA expression. Similar observations have been made by Wang and Kurpakus-Wheater (13) after exposing corneal epithelial cells for 3 days to low oxygen concentrations. They observed a 37% drop in PAI-1 mRNA levels compared to the normoxic control. Few investigations have studied the effects of reoxygenation on PAI-1 mRNA expression and they all describe declining PAI-1 mRNA levels after reoxygenation (30,31). Although these data are in accordance with some of our findings, the effect of hypoxia and reoxygenation on PAI-1 mRNA expression was different in the tumour cell lines, CAL27 and BHY. Long-term (72-h) exposure to hypoxia plus reoxygenation restored the normoxic conditions at 72 h or reached base-line levels, respectively. This was not the case for the other two cell lines. When exposed to long-term hypoxia, these cells were impaired by the low-oxygen treatment regarding PAI-1 mRNA expression and therefore did not resume PAI-1 mRNA expression, even after re-exposure to normoxic conditions.

In line with our data, augmented uPAR mRNA levels have been observed by others in human microvascular endothelial (16) and breast cancer cells (19,29) after hypoxic exposure. A possible explanation for the strong rise in uPAR mRNA during hypoxia has been supplied by the experiments of Maity and Solomon (19). They observed increased uPAR mRNA stability in breast carcinoma cells exposed to extreme hypoxia (0.2% O_2) which rapidly decreased after reoxygenation. However, upon reoxygenation, with few exceptions, we generally observed only marginal changes relative to the hypoxic situation, indicating decelerated uPAR mRNA degradation.

Different pathways are responsible for the activation of transcription after hypoxia. During hypoxia, mitochondria cannot carry out oxidative phosphorylation pathways, and electron carriers which would normally be oxidised are reduced. These reduced carriers can react directly with any existing oxygen and generate free radicals which in turn can activate transcription factors, such as HIF-1, AP-1 and NF-κB (32). Recent *in vitro* and *in vivo* studies (33,34) have shown that the reoxygenation of hypoxic tumour cells can also result in free radical formation, leading to the nuclear accumulation of Hypoxia-Inducible Factor-1 (HIF-1), a major oxygen homeostasis regulator. During normoxia, this heterodimeric transcription factor is rapidly degraded by the proteasome (34). Hypoxia, however, stabilises its hypoxia-regulated subunit, HIF-1α, thus enabling the dimerisation with its oxygen-independent subunit, HIF-1ß, to form the active HIF-1 transcription complex. This complex can then bind to hypoxia-regulatory-elements (HRE) in the nucleus, resulting in the transcription or transactivation of genes, such as PAI-1, uPA and uPAR (20,35,36). This notion is supported by certain studies showing that the stabilisation of HIF-1α is responsible for the hypoxia-mediated increased PAI-1 expression in human keloid fibroblasts and lung cancer cells (37,38). The increase in uPA, uPAR and PAI-1 mRNA levels upon reoxygenation could therefore be caused by the nuclear

accumulation of the HIF-1 complex in response to reactive oxygen during reoxygenation.

We also correlated uPA, PAI-1 and uPAR mRNA expression levels with the protein levels of these proteolytic factors determined by commercially available ELISAs and found no uniform correlation pattern. Although in agreement with the results obtained for the SCCHN cell lines, FaDu and CAL27, our findings with the SCCHN cell line, BHY, contrast with the results from a study by Spyratos *et al*. This study employed real-time quantitative PCR and ELISA measurements in order to determine uPA and PAI-1 mRNA and antigen content in breast cancer tumour specimens and found that mRNA expression data correlated with those obtained by ELISA (39). In contrast, a study correlating PAI-1 mRNA levels obtained by means of real-time RT PCR with PAI-1 antigen levels in endometrial tissue samples, only found a significant correlation in healthy tissue samples, and no correlation in ovarian endometriosis samples (40).

In our study, both hypoxia and reoxygenation induced statistically significant changes in mRNA and protein levels in the various cell lines investigated, showing that oxygen tension is a strong modulator of the plasminogen activation system *in vitro*. Additionally, various regulatory elements in the mRNAs of the components of the plasminogen activation system, such as AU-rich elements (ARE) and mRNA binding proteins, indicate that the post-transcriptional regulation of gene expression, which could lead to a higher translation efficiency, plays an important role in this system (41). As we found no uniform correlation pattern between mRNA and protein levels, we believe that in the clinical situation the determination of protein content in tumour samples is of higher diagnostic value than the determination of mRNA, since mRNA represents only a snap-shot at a particular time-point, in contrast to the final effector protein.

Oxygen tension could be a modulator of the fragile balance between the various components of the plasminogen activation system. In a solid tumour this could have various consequences: A shift towards uPA could trigger a proteolytic cascade resulting in increased tumour matrix disruption and unfavourable conditions for neovascularization (42). Increased proteolytic activity can induce autoproteolysis of plasmin and lead to the formation of angiostatin resulting in the inhibition of vascular endothelial cell proliferation and induction of endothelial cell apoptosis (43). Furthermore, a shift of balance towards PAI-1 can result in facilitated tumour cell dissemination to other organ sites since PAI-1 regulates cell adhesion and the detachment of cells from the extracellular matrix (ECM) by binding to the ECM-protein, vitronectin (44). Moreover, due to its capacity to inhibit apoptosis both in transformed and non-transformed cells (45), its ability to modify tumour angiogenesis in a concentration-dependent manner (46), and its influence on signal transduction and cell proliferation upon internalisation with its complexing partners, uPA and uPAR (9), PAI-1 can increase the aggressiveness of a tumour. In addition, *in vitro* studies have shown that the invasive capacity of tumour cells is strongly dependent on a critical balance between uPA and its inhibitor, PAI-1 (47). This highly complex interaction between the plasminogen activation system and oxygen tension could therefore explain in part why certain reoxygenated subgroups

of SCCHN tumours are associated with poor treatment outcome.

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