Reactive oxygen species-linked regulation of the multidrug resistance transporter P-glycoprotein in Nox-1 overexpressing prostate tumor spheroids

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Abstract **Expression of the multidrug resistance (MDR) transporter P-glycoprotein (P-gp) has been demonstrated to be regulated by hypoxia-inducible factor-1α (HIF-1α) and inhibited by intracellular reactive oxygen species (ROS). Herein, P-gp and HIF-1α expression were investigated in multicellular prostate tumor spheroids overexpressing the ROS-generating enzyme Nox-1 in comparison to the mother cell line DU-145. In Nox-1-overexpressing tumor spheroids (DU-145Nox1) generation of ROS as well as expression of Nox-1 was significantly increased as compared to DU-145 tumor spheroids. ROS generation was significantly inhibited in the presence of the NADPH-oxidase antagonists diphenylen-iodonium chloride (DPI) and 4-(2-amino-ethyl)benzenesulfonyl fluoride (AEBSF). Albeit growth kinetic of DU-145Nox1 tumor spheroids was decreased as compared to DU-145 spheroids, elevated expression of Ki-67 was observed indicating increased cell cycle activity. In DU-145Nox1 tumor spheroids, expression of HIF-1α as well as P-gp was significantly decreased as compared to DU-145 spheroids, which resulted in an increased retention of the anticancer agent doxorubicin. Pretreatment with the free radical scavengers vitamin E and vitamin C increased the expression of P-gp as well as HIF-1α in Nox-1-overexpressing cells, whereas no effect of free radical scavengers was observed on mdr-1 mRNA expression. In summary, the data of the present study demonstrate that the development of P-gp-mediated MDR is abolished under conditions of elevated ROS levels, suggesting that the MDR phenotype can be circumvented by modest increase of intracellular ROS generation.**
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Keywords: P-glycoprotein; NADPH oxidase; Nox-1; Reactive oxygen species; Multicellular tumor spheroid; Hypoxia-inducible factor-1α

1. Introduction

Development of multidrug resistance (MDR) mainly through overexpression of MDR transporters belonging to the ABC cassette family of transporters, e.g. P-gp, MDR-associated protein (MRP), lung resistance protein, and breast cancer-related protein is currently the main cause for the failure of chemotherapeutic cancer treatment [3,15,16]. We have recently demonstrated that intrinsic expression of P-gp in three-dimensional tissues of multicellular tumor spheroids is correlated to hypoxia in central areas of the tumor tissue [28,31]. This may correspond to the well known chemoresistance of hypoxic tumors which results in a poorer prognosis of solid tumors with hypoxic regions than their well-oxygenated counterparts [12]. Consequently, we and others showed that P-glycoprotein (P-gp) expression is regulated by the transcription factor HIF-1α which is induced under conditions of hypoxia [8,31]. Under normoxia the HIF-1α subunit is subjected to oxygen-dependent ubiquitination and proteasomal degradation that is mediated by the von Hippel–Lindau protein [20]. HIF-1α ubiquitination and degradation might also be regulated by redox modifications of the protein. Iron regulatory protein 2 is targeted for ubiquitination and proteasomal degradation by oxidative modifications that occur in the presence of iron and oxygen [14].

Low levels of reactive oxygen species (ROS) have been implicated as intracellular signaling molecules in cellular processes such as proliferation, apoptosis, and senescence [19]. It is known for many years that cancer cells own the capacity to endogenously generate ROS to significant amounts which may be one or the only cause of their excessive growth [23]. We have previously shown that small exponentially growing tumor spheroids are active in ROS generation which is downregulated upon development of quiescent cell layers and initiation of MDR in the depth of large tumors [32]. Recently, it was demonstrated that the superoxide-generating oxidase Nox-1 is functionally required for ras oncogene transformation [17]. Furthermore, it was shown that NOX5 NADPH oxidase regulates growth and apoptosis in DU-145 prostate cancer cells [4]. The present study was undertaken to evaluate whether overexpression of Nox-1 with the consequence of elevated intracellular ROS levels would affect HIF-1α and P-gp levels as well as the development of a MDR phenotype. P-gp expression has been recently demonstrated by us and others to be regulated by exogenously added prooxidants [9,24,32]. Apparently, low levels of ROS downregulate P-gp expression whereas high concentrations of prooxidants which cause oxidative stress result in

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upregulation. The latter feature may be related to the anti-apoptotic properties of the P-gp transporter [5]. Our data show that overexpression of Nox-1 in DU-145 prostate cancer cells downregulated HIF-1α as well as P-gp expression, and prevented the development of a MDR phenotype. This effect is reversed in the presence of antioxidants which result in upregulation of HIF-1α as well as P-gp expression. The data of the present study shed new light on the ROS-linked regulation of P-gp, and should be considered in approaches that use anti-oxidants for cancer treatment in patients.

2. Materials and methods

2.1. Culture technique of multicellular tumor spheroids

The human prostate cancer cell line DU-145 as well as the DU-145Nox1 cell line overexpressing Nox-1 [1] were used throughout the whole study. The cell lines were grown routinely in 5% CO_2, humidified air at 37 °C with Ham's F-10 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Sigma, Deisenhofen, Germany), 2 mM glutamine, 0.1 mM β-mercaptoethanol, 2 mM minimal essential medium, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Karlsruhe, Germany). Tumor spheroids were grown from single cells. Cell monolayers were enzymatically dissociated with 0.1% trypsin, 0.05% EDTA, and seeded in siliconized 250-ml spinner flasks (Integra Biosciences, Fernwald, Germany) with 250 ml of complete medium and agitated at 20 rotations per minute using a Cell-spin stirrer system (Integra Biosciences). Cell culture medium was partially (100 ml) changed every day.

2.2. Apoptosis and cell vitality assay

Apoptosis was assessed by annexin V staining using FITC-labeled annexin V (BD Pharmingen, San Diego, CA). Briefly, cells were incubated with 2 μl/ml annexin V solution for 15 min and fluorescence recorded at 488-nm excitation. For positive controls, cells were treated for 8 h with 2% dimethyl sulfoxide (DMSO). Cell lethality was assessed by the use of the lethal dye Sytox green (Molecular Probes, Eugene, OR) which labels the cell nuclei of dead cells with compromised cell membranes. For positive controls, cells were fixed with methanol prior to labeling with 100 nM Sytox green for 15 min. Sytox green fluorescence was excited at 488-nm.

2.3. Measurement of ROS generation

Intracellular ROS levels were measured using the fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) (Molecular Probes, Eugene, OR), which is a non-polar compound that is converted into a non-fluorescent polar derivative (H$_2$DCF) by cellular esterases after incorporation into cells. H$_2$DCF is membrane impermeable and is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS. For the experiments, multicellular tumor spheroids were incubated in E1 medium (containing in mM: NaCl 135, KCl 5.4, CaCl$_2$ 1.8, MgCl$_2$ 1, glucose 10, HEPES 10 (pH 7.4 at 23 °C)), and 20 μM H$_2$DCF-DA dissolved in DMSO was added. After 20 min, intracellular DCF fluorescence (corrected for background fluorescence) was evaluated in 3600 μm^2 regions of interest using an overlay mask unless otherwise indicated. For fluorescence excitation, the 488-nm band of the argon ion laser of a confocal laser scanning microscope (LSM410, Carl Zeiss, Jena, Germany) was used. Emission was recorded using a longpass LP 515-nm filter set.

2.4. Immunohistochemistry

Immunohistochemistry was performed with whole mount multicellular tumor spheroids. As primary antibodies the mouse polyclonal anti-mdr1 (clone Ab1) (Calbiochem, Bad Soden, Germany) (concentration of 2 μg/ml), the mouse monoclonal anti-HIF-1α antibody (BD Biosciences, Heidelberg, Germany) (dilution 1:200), the goat anti-human Nox1 (Santa Cruz Biotechnology, Santa Cruz, CA), and the mouse anti-human Ki-67 (Sigma) were used. Prior to incubation with primary antibodies tissues were fixed in 4% paraformaldehyde for 60 min at 4 °C and washed with PBS containing 1% Triton X-100 (PBST). Blocking against unspecific binding was performed for 60 min with 10% fat free milk powder (Heirler, Radolfzell, Germany) dissolved in PBST (0.01%). For P-gp staining, the tissues were subsequently incubated for 120 min at room temperature with primary antibodies dissolved in PBST (0.01%) supplemented with 10% milk powder. The tissues were thereafter washed three times with PBST (0.01% Triton) and reincubated with either a Cy3-conjugated goat anti-rabbit IgG (H + L) (P-gp), a Cy2-conjugated goat anti-mouse IgG (HIF-1α), a Cy5-conjugated donkey anti-goat (Nox-1) or a Cy5-conjugated goat anti-mouse IgG (Ki-67) (all from Dianova, Hamburg, Germany) at a concentration of 3.8 μg/ml in PBS containing 10% milk powder. After washing three times in PBST (0.01% Triton), the tissues were stored in PBST (0.01%) until inspection. For the excitation of the Cy2 fluorochrome, the 488-nm band of a helium/neon laser of the confocal setup was used. Emission was recorded using a 515-nm longpass filter set. The Cy3 fluorochrome was excited by the 543-nm band of a helium/neon laser and emission was recorded using a 570-nm longpass filter set. The Cy5 fluorochrome was excited with the 633-nm band of a helium/neon laser and emission recorded using a longpass 655-nm filter set.

2.5. Doxorubicin uptake experiments

Tumor spheroids were incubated in F10 cell culture medium supplemented with 10 μM doxorubicin for 4 h either in the presence of absence of the P-gp-reversing agent cyclosporin A (10 μM). Doxorubicin fluorescence was excited by the 543-nm line of a helium/neon laser of the confocal setup. Emission was recorded using a LP 570-nm filter set.

2.6. Quantitative RT-PCR

Total RNA from homogenized DU-145/DU-145Nox1 multicellular tumor spheroid samples was prepared using Trizol (Invitrogen) according to the manufacturer's recommendations followed by genomic DNA digestion using DNAse I/amp. grade (Invitrogen). Total RNA concentration was determined by OD$_{260\,nm}$ method. cDNA synthesis was carried out using SuperScript II RTase Kit (Invitrogen) and random hexamer primer according to the manufacturer's recommendations.

Primers were designed using the free online tool primer 3 (http://fro-do.wi.mit.edu/cgibin/primer3/primer3_www.cgi) and analyzed using netprimer (Premier Biosoft; http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The forward sequence of *mdr-1* was 5′-AAG-GAAGCCAATGCCTATGA-3′ and the reverse sequence 5′-AC-CACTGCTTCGCTTTCTGT-3′.

PCR was performed in an MJResearch Opticon II in 96-well microtitre plates using QuantiTect SYBR Green PCR kit according to the manufacturer's recommendations. In brief, cDNA product of 25 ng total RNA was mixed with QuantiTect SYBR Green PCR mastermix. Primers were added to a final concentration of 0.3 μM and water was added to a final volume of 20 μl. Amplifications were performed starting with a 15-min template denaturation/hot start step at 95 °C, followed by 40 cycles of denaturation at 94 °C for 30 s, primer annealing for 40 s at 59 °C, and extension for 30 s at 75 °C. Fluorescence increase of SYBR Green was automatically measured after each extension step. The last cycle was followed by a melting curve analysis step from 55 to 95 °C.

Each test was carried out three times. C_T values were automatically obtained. PCR efficiency was determined using the LinReg applet (http://www.gene-quantification.de/ramakers-2003.pdf). Relative expression values were obtained by normalizing C_T values of the tested genes with C_T values of the housekeeping gene RNA-Polymerase II (hRPOLII) using the C_T Method [18].

2.7. Statistical analysis

Data are given as mean values ± S.E.M. with *n* denoting the number of experiments unless otherwise indicated. In each experiment, at least 30 tumor spheroids were analyzed. Student's *t* test for unpaired data were applied as appropriate. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Overexpression of Nox-1 in DU-145 cells increases ROS generation of multicellular tumor spheroids

Du-145 prostate cancer cells were transfected with Nox-1 as previously described [1]. Nox is a homologue of gp91$phox$, the catalytic subunit of the phagocyte superoxide-generating NADPH oxidase which constitutively produces both superoxide and hydrogen peroxide when overexpressed in fibroblasts [22]. After cultivating DU-145Nox1 cells as multicellular tumor spheroids increased expression of Nox-1 protein as quantified by semiquantitative immunohistochemistry was observed as compared to the mother cell line DU-145 (Fig. 1A) ($n = 3$). The highest levels of Nox-1 expression was found in small 3 to 6-day-old tumor spheroids, whereas expression decreased in larger tumor spheroids which are known to develop central areas of quiescent, cell-cycle-inactive cells [30]. Downregulation of the expression of NADPH oxidase subunits p47-phox and p67-phox in the parental DU-145 cell line with increasing tumor spheroid size has been previously shown by us [32]. Concomitant to the elevated Nox-1 expression DU-145Nox1 tumor spheroids displayed significantly increased ROS generation as evaluated by use of the redox-sensitive fluorescence dye

H_2DCFDA. ROS generation was significantly inhibited by the non-specific NADPH oxidase inhibitors DPI (10 μM) and AEBSF (100 μM) ($n = 3$) (see Fig. 1B).

3.2. Overexpression of Nox-1 in DU-145 cells increases proliferation but not growth of multicellular tumor spheroids

It has been previously shown that H_2O_2 generated upon expression of Nox-1 in NIH 3T3 fibroblasts mediated cell growth and transformation [2]. Furthermore, cell proliferation in vitro as well as tumor growth after implantation in mice was increased in Nox-1 overexpressing DU-145 [1]. We have previously demonstrated that P-gp expression increases with the emergence of quiescent, cell-cycle-inactive cell areas which develop with prolonged cell culture of tumor spheroids [28]. It was therefore hypothesized that cell proliferation as investigated by the expression of the proliferation-associated marker Ki-67 would be increased in DU-145Nox1 tumor spheroids at different times of tumor spheroid culture. In parallel to the analysis of Ki-67 expression growth curves of tumor spheroids of the parent cell line DU-145 and DU-145Nox1 tumor spheroids were determined. According to our assumptions, Ki-67 expression was significantly increased in DU-145Nox1 tumor spheroids as compared to DU-145 tumor spheroids in all investigated ages of tumor spheroid culture and decreased with prolonged cell culture times (Fig. 2A) ($n = 3$). However, when tumor spheroid growth was assessed, the tumor spheroid volumes of DU-145Nox1 spheroids were significantly decreased as compared to DU-145 spheroids of the respective age (Fig. 2B) ($n = 3$). This is likely due to apparent shaling of loose peripheral cells from the surface of DU-145Nox1 tumor spheroids by shear stress occurring during spinner flask culture (data not shown).

To investigate whether peripheral cells shaled from the surface of DU-145Nox1 tumor spheroids were vital, apoptosis in single cells was assessed by Annexin V labeling and cell death by staining with the lethal dye Sytox green, which is cell membrane impermeant and accumulates in cell nuclei of cells with compromised cell membranes. When single cells shaled from the periphery of tumor spheroids were plated to coverslips, they attached to the glass surface within minutes, started to proliferate after few hours, and displayed intracellular calcium responses after stimulation with ATP (data not shown). As shown in Fig. 3, the cells were annexin V and Sytox green negative, indicating that they were vital with no signs of apoptosis.

3.3. Nox-1 overexpressing cells display reduced protein levels of P-gp

The working hypothesis of the present study suggests that elevated ROS generation in DU-145Nox1 tumor spheroids prevents expression of the MDR transporter P-gp which was previously demonstrated to be upregulated in large, cell-cycle-inactive DU-145 tumor spheroids displaying decreased ROS generation [28,32]. To evaluate this issue, P-gp expression was assessed during the growth of DU-145 as well as DU-145-Nox1 tumor spheroids by semiquantitative immunohistochemistry. As previously reported P-gp expression was transiently increased during tumor spheroid growth of DU-145 cells with a maximum occurring around day 10 of cell culture. A comparable time course of P-gp expression was likewise obtained in DU-145Nox1 tumor spheroids. However, the expression level of P-gp in DU-145Nox1 spheroids was significantly
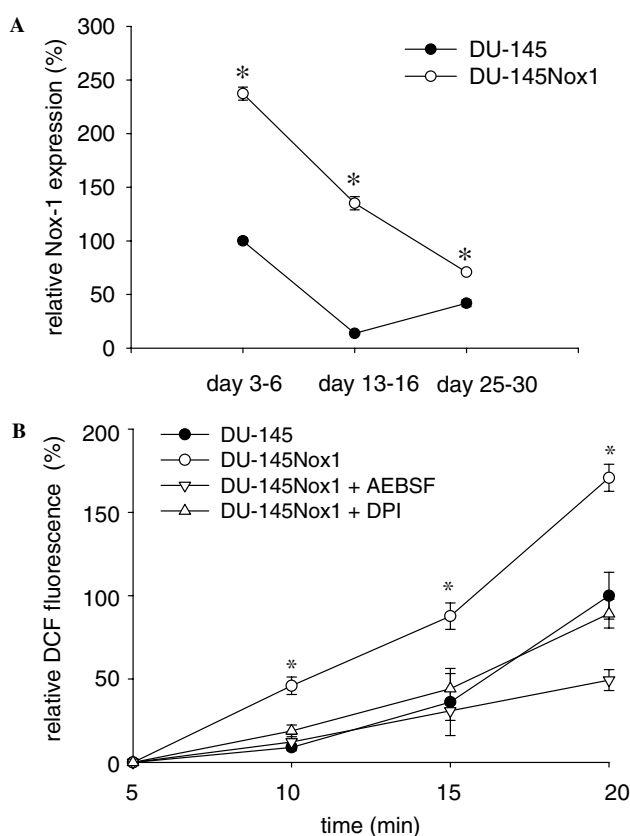

Fig. 1. Increased Nox-1 expression (A) and generation of ROS (B) in multicellular DU-145 and DU-145Nox1 tumor spheroids. Nox-1 expression was evaluated in 3–6-, 13–16-, and 25–30-day-old tumor spheroids by semiquantitative immunohistochemistry. Intracellular ROS were assessed by use of the redox-sensitive indicator H_2DCF-DA in the absence or presence of the NADPH oxidase inhibitors DPI (10 μM) and AEBSF (100 μM). Note, that Nox-1 expression decreased with increasing age of tumor spheroids. $^*P < 0.05$ significantly different as compared to the DU-145 tumor spheroids.
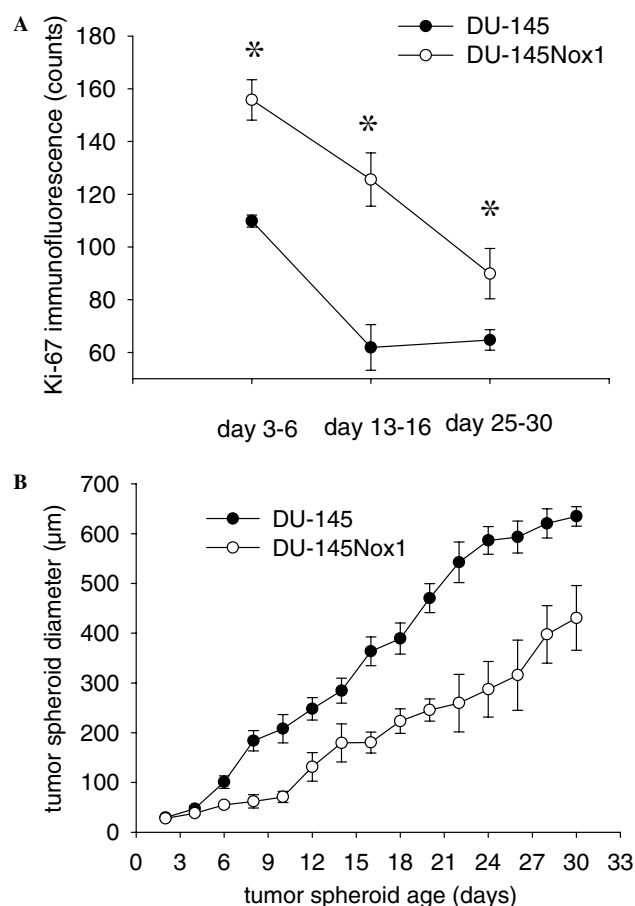
Fig. 2. Expression of the proliferation marker Ki-67 (A) and growth (B) of DU-145 and DU-145Nox1 multicellular tumor spheroids. Ki-67 expression was significantly increased in all age classes of DU-145Nox1 tumor spheroids under investigation but decreased during the growth of tumor spheroids. The size of DU-145Nox1 tumor spheroids was decreased as compared to the parental cell line which is presumably due to the shaling of loosely attached cells from the surface of DU-145Nox1 tumor spheroids. $^*P < 0.05$ significantly different as compared to the DU-145 tumor spheroids.

lower in all ages of tumor spheroids under investigation (Fig. 4) ($n = 30$ tumor spheroids in each data point).

3.4. Overexpression of Nox-1 in DU-145 prevents induction of the MDR phenotype

The decreased expression level of P-gp in DU-145Nox1 tumor spheroids should result in the development of a MDR phenotype, i.e. in reduced drug uptake due to P-gp-mediated drug export. To investigate development of a MDR phenotype, multicellular tumor spheroids were incubated with the P-gp substrate doxorubicin (10 μM), and doxorubicin uptake was evaluated in the absence and presence of the P-gp-reversing agent cyclosporin A (10 μM) after 4 h of incubation (Fig. 5) ($n = 3$). Doxorubicin fluorescence was significantly decreased in DU-145 tumor spheroids as compared to DU-145Nox1 spheroids which is due to increased doxorubicin export. As previously shown [32] incubation with the P-gp-reversing agent cyclosporin A significantly increased doxorubicin retention in the parent DU-145 cell line indicating the presence of P-gp-mediated MDR in DU-145 prostate tumor spheroids ($n = 3$). In contrast, doxorubicin uptake in the pres-

ence of cyclosporin A was not significantly increased in DU-145Nox1 tumor spheroids which is presumably due to the decreased expression level of P-gp in this cell line.

3.5. Upregulation of HIF-1α and P-gp upon incubation of DU-145Nox1 tumor spheroids with free radical scavengers

The data of the present study demonstrate that Nox-1 overexpression prevents the development of the MDR phenotype which is observed in large tumor spheroids of the parental cell line DU-145. Since we and others have previously shown that P-gp expression is under the control of HIF-1α [8,31] we assumed that HIF-1α would likewise be decreased in the DU-145Nox1 cell line. Furthermore, preincubation with free radical scavengers should result in upregulation of HIF-1α as well as P-gp expression. To verify the assumption that the decreased expression of HIF-1α as well as P-gp in DU-145Nox1 tumor spheroids was due to increased ROS generation in DU-145Nox1 cells, tumor spheroids were incubated from day 6 to day 14 with the free radical scavengers vitamin C (30 μM) and vitamin E (100 μM). Subsequently P-gp as well as HIF-1α expression was assessed (Fig. 6A and B). Preincubation for 7 days in vitamin C/E containing cell culture medium significantly increased HIF-1α (see Fig. 6A) as well as P-gp (see Fig. 6B) expression in the DU-145Nox1 tumor spheroids but not in the DU-145 tumor spheroids. This clearly indicates that the increased ROS levels in DU-145Nox1 tumor spheroids prevented the development of the P-gp-mediated MDR phenotype.

To investigate whether incubation with free radical scavengers changed mRNA expression, quantitative RT-PCR was performed with tumor spheroids of the DU-145 as well as the DU-145Nox1 cell line. It was observed that incubation with free radical scavengers did not significantly increase *mdr-1* mRNA expression in DU-145 as well as DU-145Nox1 tumor spheroids which strongly suggests that the effects of ROS on P-gp expression are due to changes in protein turnover (Fig. 7) ($n = 3$).

4. Discussion

Previous studies of our group have demonstrated that low concentrations of exogenously added prooxidants downregulated P-gp expression and reversed the MDR phenotype in DU-145 prostate tumor spheroids [26,31]. The MDR phenotype in tumor spheroids of different origin was shown to develop at times when the exponential growth of tumor spheroids attains a plateau phase and quiescent, cell-cycle-inactive cell areas are present in the deeper layers of the tumor tissue which is paralleled by the development of central hypoxia and downregulation of ROS generation [28,32]. The coherence between elevated ROS generation and cell-cycle progression is meanwhile well established. It has been shown in a variety of studies that ROS play a role in many growth factor- and cytokine-mediated signaling cascades [19]. Furthermore, the aggressive growth of a variety of tumor cells is well known to be associated with increased generation of ROS [23]. Transformation of NIH 3T3 cells with constitutively active *Ras* resulted in elevated ROS generation and increased cell-cycle activity suggesting ROS as mediators of Ras-induced cell-cycle progression [13]. Additionally, overexpression of the gp91*phox* homologue
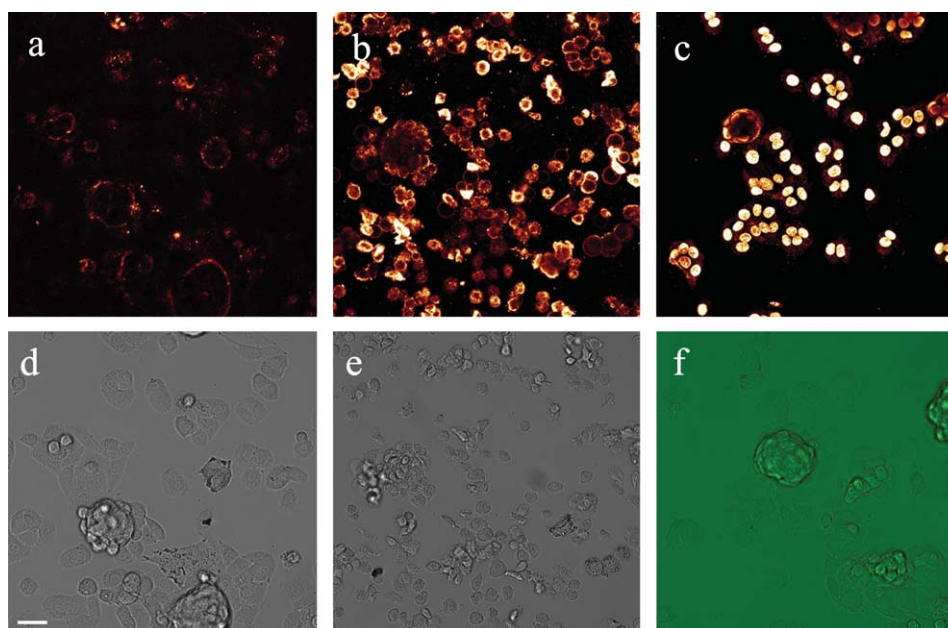
Fig. 3. Cell vitality of single cells shaled from the periphery of DU-145Nox1 tumor spheroids. Apoptosis was assessed by annexin V staining and cell lethality by labeling of cell nuclei with Sytox green. Annexin V labeling was negligible in control tumor spheroids (a), indicating absence of apoptosis, whereas a prominent staining was observed when apoptosis was induced by DMSO (1%) treatment for 8 h (b) (d, e represent transmission images of a, b, respectively). Systox green staining was absent in control cells, as shown in an overlay between a transmission image and a Sytox green fluorescence image (f), indicating that cells were vital. In contrast, staining of cell nuclei was obvious when cells were killed by methanol fixation (c). The bar represents 20 μm.
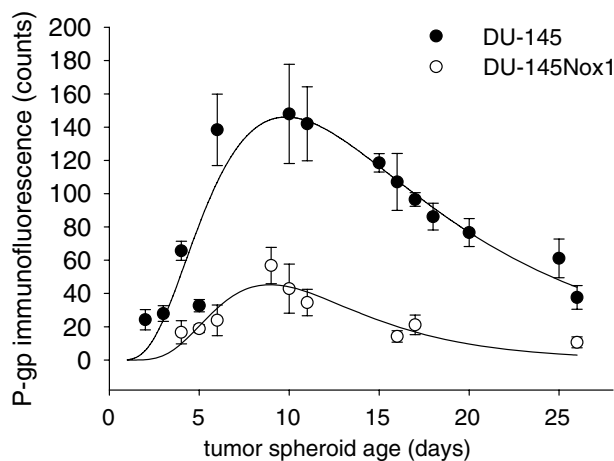

Fig. 4. Expression of P-gp during the growth of DU-145 and DU-145Nox1 tumor spheroids as evaluated by semiquantitative immuno-histochemistry. P-gp displayed transient expression in tumor spheroids of both cell lines. However, P-gp was significantly decreased in DU-145Nox1 tumor spheroids of all ages under investigation.

Nox-1 in NIH 3T3 cells led to increased superoxide generation, increased mitotic rate, cell transformation and tumorigenicity [2]. In previous studies, we have shown that during the exponential growth phase of tumor spheroids the cells are expressing NADPH oxidase and robustly generate ROS. Upon induction of cell quiescence and decay in tumor growth ROS generation as well as expression of NADPH oxidase was downregulated [32]. These previous observations led to the hypothesis that expression of P-gp is linked to the intracellular ROS level of the tumor cells.

In the present study, it was shown that DU-145Nox1 tumor spheroids displayed elevated levels of ROS as compared to the parent cell line DU-145. Nox-1 expression in DU-145Nox1 tumor spheroids decreased with prolonged cell culture time which was paralleled by downregulation of the proliferation marker Ki-67. Comparable effects were obtained in the parental cell line DU-145 albeit the expression of Nox-1 as well as Ki-67 was at a lower level as compared to DU-145Nox1 tumor spheroids at each age class investigated. This indicates that NADPH-oxidase expression is closely associated with cell-cycle activity of the tumor cells. In this respect, it has been recently demonstrated that Nox-2 deficiency in mice led to reduced cellular proliferation and leukocyte accumulation [6]. The control of P-gp expression by the proliferation status of cells is still highly controversial. Previously, it has been shown that MDR is expressed on cell-cycle-inactive human hemato-poietic progenitors and leukemic blasts. Proliferation induction of the cells resulted in increased anthracycline sensitivity, i.e. reversal of the MDR phenotype [21]. In a further study, it was shown that P-gp turnover is a cell-cycle-related process in MDR cells since P-gp half-life was considerably increased when the cell cycle of cancer cells was delayed in the G0/G1 phase [34]. These data were corroborated by a recent study of us which demonstrated that experimental arrest of DU-145 tumor spheroids in the G0/G1 phase resulted in parallel upregulation of P-gp and the cyclin-dependent kinase inhibitor p27^{kip1} [27].

The working hypothesis of the present study assumed that the increased ROS generation in DU-145Nox1 tumor spheroids would depress P-gp expression. To verify this assumption P-gp expression was monitored during the growth of DU-145Nox1 and DU-145 tumor spheroids. Although a transient expression of P-gp was observed in DU-145Nox1 as well as
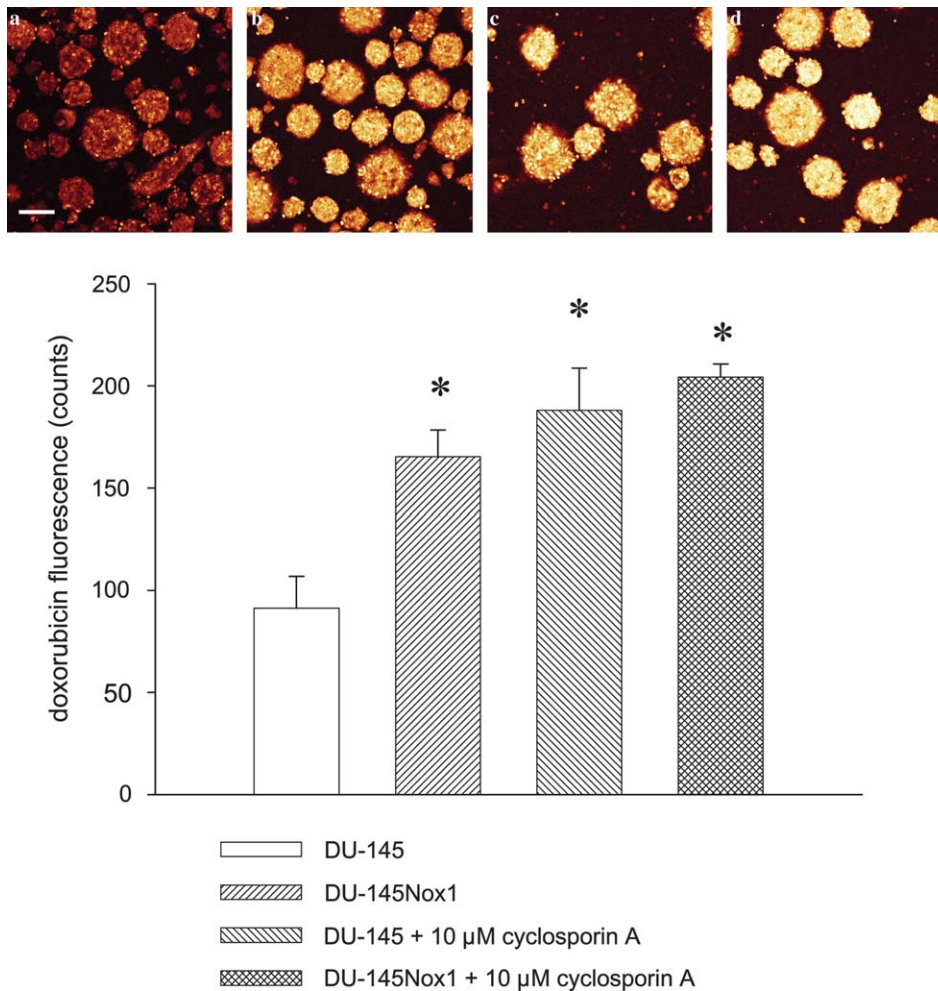
Fig. 5. Uptake of the P-gp substrate doxorubicin (10 μM) in tumor spheroids of the DU-145 and DU-145Nox1 cell line. Doxorubicin (10 μM) uptake after 4 h was significantly lower in DU-145 tumor spheroids as compared to DU-145Nox1 tumor spheroids, indicating absence of a MDR phenotype in the latter cell line. Preincubation with the P-gp-reversing agent cyclosporin A (10 μM) increased doxorubicin uptake in drug resistant DU-145 tumor spheroids but not in drug-sensitive DU-145Nox1 spheroids. The bar represents 200 μm. $^*P < 0.05$ significantly different as compared to the DU-145 tumor spheroids.

in DU-145 tumor spheroids the expression level of P-gp was significantly decreased in the DU-145Nox1 cell line as compared to DU-145 cells, which strongly suggests that P-gp expression is regulated by the intracellular ROS level. Increased P-gp expression should result in the development of a MDR phenotype resulting in decreased drug uptake. Indeed, it was observed that doxorubicin uptake was decreased in DU-145 tumor spheroids as compared to spheroids of the DU-145Nox1 cell line. Addition of the P-gp antagonist cyclosporin A efficiently reversed the MDR phenotype, whereas no significant increase in doxorubicin uptake was observed in DU-145Nox1 tumor spheroids. This clearly indicates that P-gp is functional and renders DU-145 tumor spheroids drug resistant.

If the elevated ROS levels in DU-145Nox1 tumor spheroids are the cause for the observed decreased P-gp levels, neutralization of ROS by free radical scavengers should reverse the observed effects. Furthermore, since it has been previously demonstrated that the mdr-1 gene is under the control of HIF-1α [8,31] this transcription factor should be downregulated in DU-145Nox1 tumor spheroids. Indeed, it was demon-strated that DU-145Nox1 tumor spheroids not only displayed decreased P-gp levels but likewise decreased levels of HIF-1α. Incubation with free radical scavengers increased HIF-1α as well as P-gp expression which clearly demonstrates that P-gp expression in multicellular prostate tumor spheroids is regulated by the intracellular ROS level. However, our data suggest that the observed affects of free radical scavengers on P-gp expression were due to a decrease in protein turnover rather than transcriptional activation since quantitative RT-PCR data demonstrated that free radical scavengers failed to significantly upregulate mdr-1 mRNA neither in DU-145Nox1 tumor spheroids nor in the parental cell line. These data are corroborated by a recent study which demonstrated that free radical scavengers did not increase HIF-1α gene expression as evaluated by the use of a luciferase reporter construct under the control of multiple HREs [7]. Since previous studies of our group demonstrated that HIF-1α as well as P-gp protein expression was increased following incubation with free radical scavengers it may be suggested that the cellular ROS level regulates HIF-1α as well as P-gp protein stability rather than gene expression [31].
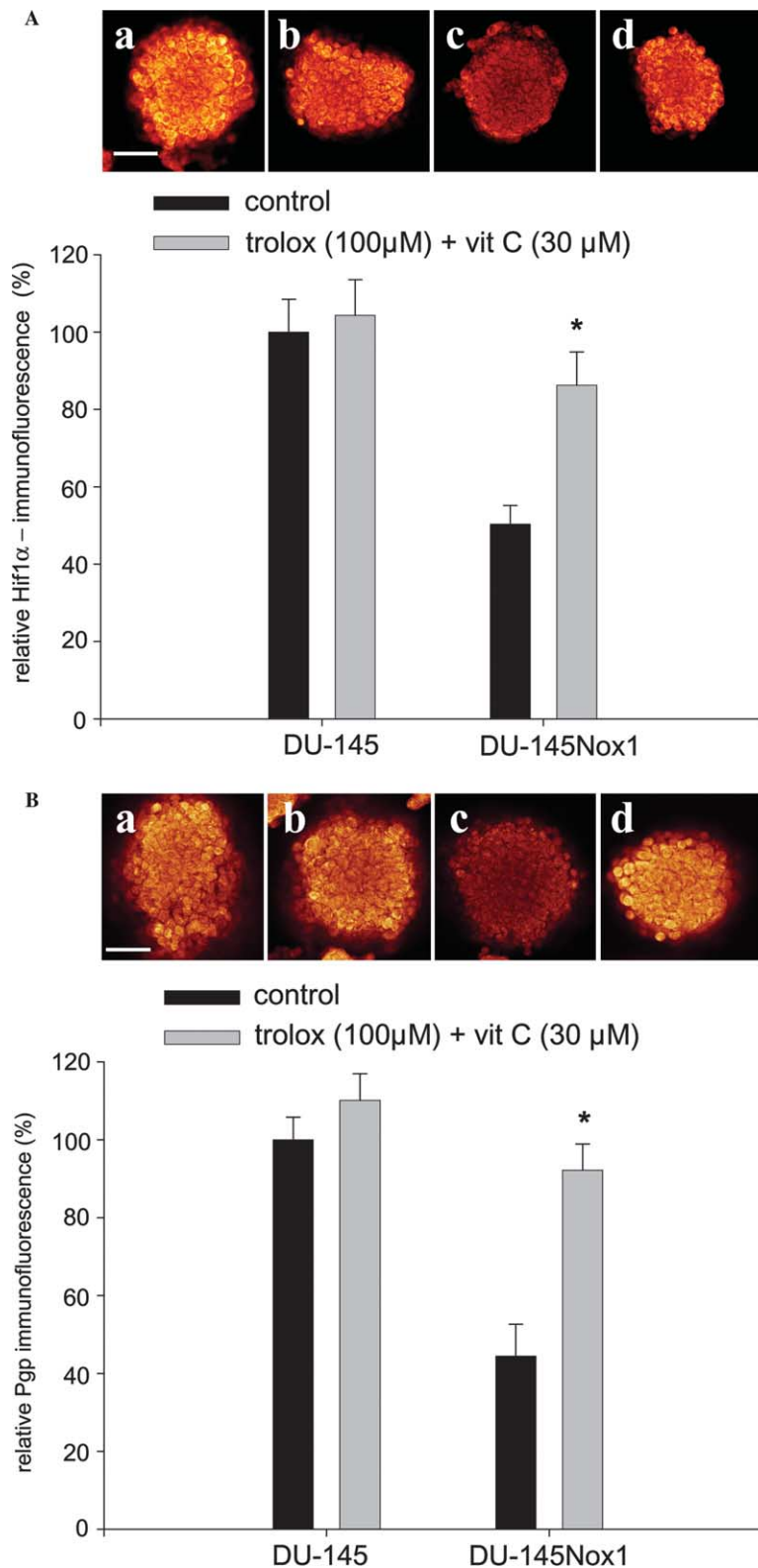
Fig. 6. Effects of the free radical scavengers vitamin C and vitamin E on HIF-1α (A) and P-gp (B) levels in DU-145 and DU-145Nox1 tumor spheroids. Tumor spheroids were treated from day 6 to day 14 with vitamin C (30 μM) and vitamin E (100 μM) and subsequently analyzed by semiquantitative immunohistochemistry for HIF-1α and P-gp expression. The representative images show: (a) DU-145 untreated; (b) DU-145 treated with vitamins; (c) DU-145Nox1 untreated; (d) DU-145Nox1 treated with vitamins. Note, that treatment with free radical scavengers significantly increased HIF-1α and P-gp in DU-145Nox-1 tumor spheroids as compared to spheroids of the parental DU-145 cell line which displays already maximum expression of HIF-1α and P-gp. The bar represents 150 μm. [*]$P < 0.05$ significantly different as compared to the untreated control.
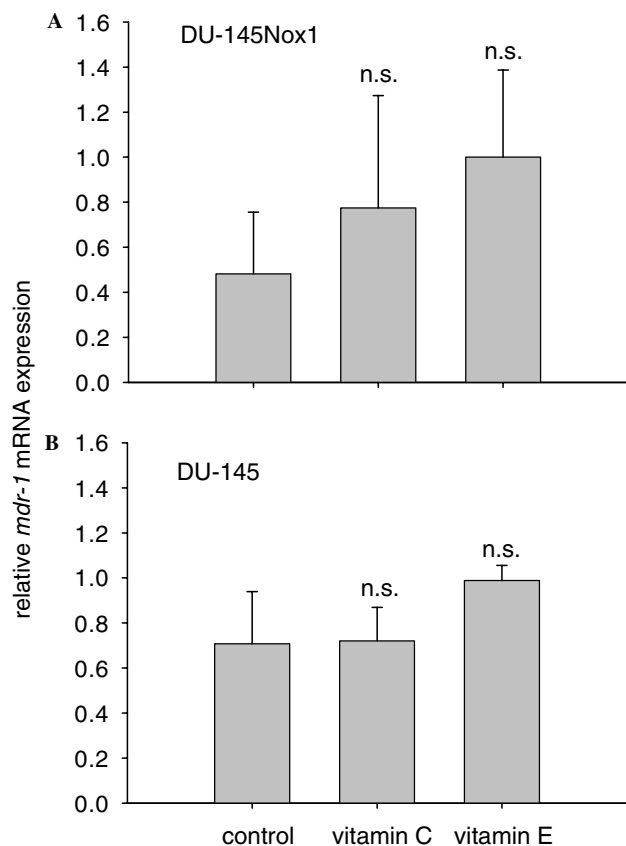
Fig. 7. Effects of the free radical scavengers vitamin C and vitamin E on *mdr-1* mRNA expression in DU-145Nox1 (A) and DU-145 (B) multicellular tumor spheroids. Note, that treatment of DU-145 as well as DU-145Nox1 tumor spheroids with free radical scavengers did not significantly increase *mdr-1* mRNA. $^*P < 0.05$ significantly different as compared to the untreated control.

The effect of ROS on P-gp expression is apparently dose-dependent. We have previously shown that low concentrations of ROS downregulated P-gp expression, whereas high concentrations which induce cell lethality after prolonged incubation resulted in P-gp increase [32]. Increased P-gp expression on the protein as well as transcriptional level following transient oxidative stress has been shown to occur in primary cultured rat brain endothelial cells [9]. Previously, it has been demonstrated that upregulation of P-gp via nuclear factor-κB activation protects kidney proximal tubule cells from cadmium- and ROS-induced apoptosis [24]. Stress-induced expression of P-gp has been shown for various conditions, e.g. UV radiation [25], low external pH, osmotic shock [33], and hyperthermia [29] which may be associated with robust elevations of intracellular ROS. ROS-mediated stress conditions have likewise been shown to be involved in the regulation of HIF-1α expression and/or stability [11]. In this respect, it was recently demonstrated that the accumulation of H_2O_2 in junD−/− cells decreases the availability of FeII and reduces the activity of HIF prolyl hydroxylases that target hypoxia-inducible factors-α (HIF-α) for degradation [10]. Oxidative stress conditions may elicit anti-apoptotic signaling pathway which are distinct from those activated by low level ROS generation and resulting in cell-cycle activation, increased tumor cell growth and downregulation of P-gp expression. In these premises, the dissection of high level versus low level ROS-medi-

ated signaling pathway appears inevitably necessary to understand the regulation of P-gp and HIF-1α expression. This is to the more important since HIF-1α not only controls P-gp expression which limits the benefit of chemotherapeutic cancer but also vascular endothelial growth factor which is a key determinant of vascularization of solid tumors during tumor-induced angiogenesis.

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