Intrinsic expression of the multidrug resistance (MDR) transporter P-glycoprotein (Pgp) may be regulated by reactive oxygen species (ROS). A transient expression of Pgp was observed during the growth of multicellular tumor spheroids. Maximum Pgp expression occurred in tumor spheroids with a high percentage of quiescent, Ki-67-negative cells, elevated glutathione levels, increased expression of the cyclin-dependent kinase inhibitors p27\textsuperscript{Kip1} and p21\textsuperscript{WAF1} as well as reduced ROS levels and minor activity of the mitogen-activated kinase (MAPK) members c-Jun amino-terminal kinase (JNK), extracellular signal-regulated kinase ERK1,2, and p38 MAPK. Raising intracellular ROS by depletion of glutathione with buthionine sulfoximine (BSO) or glutamine starvation resulted in down-regulation of Pgp and p27\textsuperscript{Kip1} whereas ERK1,2 and JNK were activated. Down-regulation of Pgp was furthermore observed with low concentrations of hydrogen peroxide and epidermal growth factor, indicating that ROS may regulate Pgp expression. The down-regulation of Pgp following BSO treatment was abolished by agents interfering with receptor tyrosine kinase signaling pathways, i.e., the protein kinase C inhibitors bisindolylmaleimide I (BIM-1) and Ro-31-8220, the p21\textsuperscript{WAF1} farnesyl protein transferase inhibitor III, the c-Raf inhibitor ZM 336372 and PD98059, which inhibits ERK1,2 activation. ROS involved as second messengers in receptor tyrosine kinase signaling pathways may act as negative regulators of Pgp expression.

The treatment of cancer is limited by either intrinsic or acquired expression of multidrug resistance transporters, including Pgp.\textsuperscript{1} The Pgp-mediated MDR phenotype is generally characterized by a decreased cellular drug accumulation owing to an enhanced drug efflux and is caused by the overexpression of the drug transporter (1–3). Most in vitro studies have been performed using cell cultures selected for drug resistance by repetitive treatment with anticancer agents or using recurrent tumors from patients after chemotherapy (3). However, there is increasing evidence that Pgp-mediated MDR is likewise developing in the multicellular context of tumor tissues, without previous treatment with anticancer agents (4–8). This intrinsic Pgp-mediated MDR may represent a so far underestimated common in vivo situation, which may even worsen the efficacy of chemotherapeutic regimen. Especially, renal and prostate cancers are known as intrinsic drug-resistant cancers, which already have a relatively resistant phenotype to anticancer agents even without drug selection (9). This may explain the low response of hormone-insensitive prostate tumors toward cytostatic agents (9, 10).

In most strategies developed to reverse the MDR phenotype, chemical-reversing agents are applied, which have in common the ability to reverse the MDR phenotype through the binding to the MDR transporters (11). A more efficient strategy to circumvent MDR would be to down-regulate the expression of genes coding for the transporters. This, however, requires knowledge of the molecular mechanisms and signal transduction pathways that are involved in the regulation of MDR-related genes and elaboration of sophisticated experimental approaches that efficiently down-regulate the drug transporters. Recently, it has been pointed out that the expression of Pgp may be redox-regulated, because down-regulation of Pgp and reversal of the MDR phenotype was achieved by treatment of tumor cells with tumor necrosis factor\textalpha (12), which is known to utilize low levels of ROS in its signal transduction pathway (13). On the other hand, it has been reported that high levels of ROS resulting in severe cellular oxidative stress increased the expression of the MDR-1b (15) genes.

In previous studies we have reported on an intrinsic Pgp MDR, which develops in quiescent cell areas of large, non-necrotic multicellular prostate tumor spheroids derived from the androgen-independent cell line DU-145 (7, 8). We furthermore demonstrated that the MDR phenotype could be reversed and that Pgp could be down-regulated upon incubation of spheroids with chemical agents that raised intracellular ROS and stimulated cell cycle activity (16). The current study presents evidence that the development of intrinsic Pgp expression is a general phenomenon occurring in multicellular tumor spheroids of different origin, i.e., melanoma, hepatoma, and glioma.

PCR, reverse transcriptase-polymerase chain reaction; ERK, extracellular signal-regulated kinase; bp, base pair(s).
Redox-regulation of Pgp

Larger, non-necrotic tumor spheroids (diameter 300 μM) were grown in minimal essential amino acids, 100 IU/ml penicillin, and 100 μg/ml streptomycin (ICN Flow, Meckenheim, Germany). Spheroids were grown from single cells. Cell monolayers were enzymatically dissociated with 0.2% trypsin, 0.05% EDTA (ICN Flow) and seeded in siliconized 250-ml spinner flasks (Integra Biosciences). Cell culture medium was partially supplemented with 10% fetal calf serum, 0.1 mM 9-[2-oxo-2-(E)- (2-oxo-2-(E)-dimethylaminobenzamido)-4-oxa-4-azaspiro(3,3)heptane]-9(M)-H2O2. After the incubation time tumor spheroids were fixed in 4% paraformaldehyde and either in ice-cold methanol/acetone (7:3) or 4% paraformaldehyde fixed in either ice-cold methanol/acetone (7:3) or 4% paraformaldehyde (excitation at 543 nm, emission, long-pass filter set). For fluorescence excitation, the 488-nm laser of the confocal setup and a long-pass LP 515-nm filter set.

Doxorubicin Fluorescence Recording and Confocal Laser Scanning Microscopy—The fluorescent cytostatic anti-cancer agent doxorubicin (Sigma Chemical Co.) (excitation at 543 nm, emission, long-pass filter set) was used at a concentration of 10 μM unless otherwise indicated. Multicellular tumor spheroids were loaded with doxorubicin at 37 °C for 90 min. After recording of doxorubicin fluorescence by confocal laser scanning microscopy, spheroids were transferred to doxorubicin-free cell culture medium and incubated at 37 °C for 30 min. Following this incubation doxorubicin retention in multicellular spheroids was evaluated.

Fluorescence recordings were performed by means of a confocal laser scanning setup (LSM 410, Carl Zeiss, Jena, Germany), connected to an inverted microscope (Axiovert 135, Carl Zeiss). Fluorophores were excited using a 0.5-milliwatt helium-neon laser (single excitation at 543 nm). A 25×, numerical aperture 0.8, oil immersion-corrected objective (Carl Zeiss) was applied. Doxorubicin fluorescence was measured in 3600-μm² areas in a depth up to 80 μm from the spheroid periphery.

**Experimental Procedures**

**Culture Technique of Multicellular Tumor Spheroids**—The human prostate cancer cell line DU-145 and the melanoma cell line IGR were grown in 5% CO₂/humidified air at 37 °C with Ham’s F-10 medium (Life Technologies, Gaithersburg, MD). The glioma cell line Gli36 and the hepatoma cell line HepG2 were grown in Dulbecco’s medium (Life Technologies, Inc.). The cell culture media were supplemented with 10% fetal calf serum, 0.1 mM 9-[2-oxo-2-(E)- (2-oxo-2-(E)-dimethylaminobenzamido)-4-oxa-4-azaspiro(3,3)heptane]-9(M)-H2O2 and 100 μg/ml streptomycin (ICN Flow, Meckenheim, Germany). Spheroids were grown from single cells. Cell monolayers were enzymatically dissociated with 0.2% trypsin, 0.05% EDTA (ICN Flow) and seeded in siliconized 250-ml spinner flasks (Integra Biosciences, Farnworth, Germany) with 250 ml of complete medium and agitated at 20 rpm using a Cell Spin stirrer system (Integra Biosciences). Cell culture medium was partially supplemented (125 ml) every day.

**Incubation with BSO, Glutamine-reduced Medium, and Ebselen**—For incubation with BSO and glutamine-reduced medium, multicellular tumor spheroids (diameter 130 ± 50 μm, day 5) were transferred to bacteriological tissue culture plates (diameter 10 cm) (Becton Dickinson, Meylan, France) filled with 10 ml of F10 cell culture medium. They were subsequently treated for 7 days with 50 μM BSO (Sigma, Deisenhofen, Germany), which was added to the cell culture medium. For incubation in glutamine-reduced medium, F10 cell culture medium was supplemented with 10% fetal calf serum and 0.2% trypsin/0.05% EDTA. An aliquot of 3.5 × 10⁶ cells was incubated for 24 h in 6-cm bacteriological Petri dishes containing 10 ml of 0.01% containing 10% fat-free milk powder to reduce nonspecific binding and for further 2 h with primary antibody. After washing three times in PBST (0.1%), the spheroids were incubated for 60 min in PBST (0.01%) supplemented with 10% milk powder and either a Cy5-conjugated F(ab)₂ fragment goat anti-mouse IgG (H+L) (concentration 3.25 μg/ml), a Cy5-conjugated F(ab)₂ fragment goat anti-rabbit IgG (H+L) (concentration 4.8 μg/ml) or a Cy5-conjugated F(ab)₂ fragment goat anti-rabbit IgG (H+L) (concentration 4.6 μg/ml) from Dianova, Hamburg, Germany. Excitation was performed using a 633-nm helium-neon laser of the confocal setup. Emission was recorded using a long-pass LP 655-nm filter set.

For quantitative immunohistochemistry, confocal images were recorded from whole mount multicellular spheroids stained with only secondary antibodies (background fluorescence image) and spheroids incubated with primary antibodies. All conditions of the confocal setup were adjusted to yield optical slices of 10-μm thickness. After subtraction of background fluorescence, the fluorescence signal (counts) was evaluated by the image analysis software of the confocal setup in 3600-μm² areas of interest and was routinely exported for further analysis to the Sigma Plot graphic software.

**Determination of the Intracellular Redox Levels**—Intracellular redox levels were measured using the fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Molecular Probes), which is a nonpolar compound that is converted into a non-fluorescent polar derivative (H₂DCF) by cellular esterases after incorporation into cells. H₂DCF is membrane-impermeable and is rapidly oxidized to the highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) in the presence of intracellular ROS (17). For the experiments, multicellular tumor spheroids were incubated in E1 medium containing (in millimolar) NaCl 135, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, glucose 10, HEPES 10 (pH 7.4 at 23 °C), and 20 μM H₂DCFDA dissolved in dimethyl sulfoxide (Me₂SO) was added. After 5, 10, 15, 20, and 30 min intracellular DCF fluorescence (corrected for background fluorescence) was evaluated in 3600-μm² regions of interest using an overlay mask. For fluorescence excitation, the 488-nm bandpass filter of the confocal setup was used. Emission was recorded using a long-pass LP515-nm filter set.

**Determination of Intracellular Glutathione**—Glutathione levels were determined using a commercially available glutathione assay kit (Calbiochem). Tumor spheroids were enzymatically dissociated in 0.1% trypsin/0.05% EDTA. An aliquot of 3.5 × 10⁶ cells was lysed in 0.5 ml of meta-phosphoric acid, freeze-thawed, and centrifuged (3000 × g) for 10
RESULTS

Transient Expression of Pgp in Multicellular Tumor Spheroids—We have previously reported on the development of an intrinsic Pgp-mediated MDR in multicellular prostate tumor spheroids of the DU-145 cell line, which paralleled the induction of cell quiescence in the depth of the multicellular tissue (7, 8). To evaluate whether the transient expression of Pgp observed in DU-145 tumor spheroids represents a general phenomenon of multicellular neoplastic tissues, tumor spheroids of different origin, i.e. melanoma tumor spheroids of the IGR cell line, hepatoma spheroids of the Hepa1 cell line, and glioma spheroids of the Gli36 cell line were screened for the expression of Pgp and compared with protein levels of Pgp in tumor spheroids of the DU-145 cell line. It was observed that all cell lines under investigation transiently expressed Pgp (Fig. 1, A–D) (n = 3). Owing to the cell-specific growth kinetics of tumor spheroids of different origin (Fig. 2) the maximum Pgp expression occurred at different times in DU-145, IGR, Hepa1, and Gli36 tumor spheroids (n = 3). In DU-145 prostate tumor spheroids the expression of the MDR-1 gene in relation to tumor spheroid age was evaluated by RT-PCR and immunoblot analysis (Fig. 3). It was evidenced that MDR-1 expression paralleled Pgp expression, i.e. it was low in small tumor spheroids and increased with the development of quiescent cell areas in 5- to 10-day-old tumor spheroids. MDR-1 expression was decreased in large tumor spheroids, which have been previously shown to contain central necrosis (27-day-old) (n = 3) (19).

To correlate the increased expression of Pgp in DU-145 tumor spheroids with the proliferation status of the tumor cells, the tissue levels of the CDK inhibitors p27Kip1 and p21WAF-1 as well as the expression of the proliferation-associated antigen Ki-67 were analyzed. It was observed that p27Kip1 (Fig. 4A) and p21WAF-1 (Fig. 4B) were up-regulated in parallel to Pgp with increasing size of tumor spheroids (n = 3). With the onset of central necrosis, which occurs at a critical size of 350 ± 50 μm in DU-145 tumor spheroids (20) p27Kip1 expression declined to the level observed in small tumor spheroids, whereas p21WAF-1 expression remained on an elevated level. Ki-67 expression was
inversely correlated to the expression of Pgp and declined with increasing age and size of tumor spheroids, indicating a decrease in proliferative activity ($n = 3$).

The apparent correlation of cell quiescence and increased Pgp expression was furthermore corroborated by investigation of the intrinsic activity of the MAPK members ERK1,2, JNK, and p38 MAPK, which are involved in mitogenic and stress-activated signaling pathways (Fig. 5). As expected, the activity of all investigated MAPK members was low in large, non-necrotic DU-145 tumor spheroids (diameter $300 \pm 50 \mu m$, days 14–15 of tumor spheroid culture), which expressed maximum levels of Pgp (see Fig. 1). Increased JNK activity was observed in small tumor spheroids (diameter $100 \pm 50 \mu m$, days 3–6 of tumor spheroid culture), as well as large tumor spheroids containing central necrosis (diameter $500 \pm 100 \mu m$, days 18–40 of tumor spheroid culture) ($n = 3$), whereas increased ERK1,2 activity was observed solely in exponentially growing small tumor spheroids. A low basal activity of p38 MAPK was observed in all size classes of tumor spheroids under investigation ($n = 3$).

**Endogenous ROS Generation and Glutathione Content in Multicellular Tumor Spheroids**—Cancer cells endogenously generate ROS (21), which are involved in signaling pathways maintaining cell proliferation (22–24). Hence, it is expected that endogenous ROS generation is high in small tumor spheroids consisting predominantly of proliferating cells, whereas it is low in large, non-necrotic, tumor spheroids, which contain extended areas of cell cycle-inactive, quiescent cells. To determine ROS generation in multicellular DU-145 tumor spheroids, small, large non-necrotic as well as tumor spheroids containing extended central necrosis were stained with the redox-sensitive dye H$_2$DCFDA, and the time course of ROS generation was monitored. Indeed our data show that the generation of ROS (evaluated after an incubation time of 20 min with H$_2$DCFDA) was most pronounced in small spheroids with $280 \pm 25\%$ of the fluorescence increase observed in large, non-necrotic tumor spheroids (set to 100%) ($n = 3$). In tumor spheroids containing central necrosis, the ROS generation was significantly resumed and amounted to $200 \pm 25\%$ of the ROS generation observed in large, non-necrotic spheroids ($n = 3$) (Fig. 6A).

Intracellular ROS in DU-145 are presumably generated via an NADPH-oxidase-like enzyme, which may be differentially expressed during the growth of tumor spheroids. Therefore, tumor spheroids were immunostained for the NADPH-oxidase subunits p47$^{\text{phox}}$ and p67$^{\text{phox}}$. It was demonstrated that small tumor spheroids exerted high levels of NADPH-oxidase expression, whereas in large non-necrotic tumor spheroids a significant down-regulation of NADPH oxidase expression was observed (Fig. 6B). In large tumor spheroids containing central necrosis, p47$^{\text{phox}}$ and p67$^{\text{phox}}$ expression was partially resumed which, however, did not reach statistical significance ($n = 3$).

Intracellular ROS levels are counterbalanced by the antioxidative defense system. Therefore, the intracellular content of glutathione which is an important cellular anti-oxidative thiol, was determined in tumor spheroids at different times of tumor spheroid culture (Fig. 6C). Our data demonstrate that the glutathione content was inversely correlated to ROS levels in tumor spheroids. Low levels of glutathione were determined in small tumor spheroids ($6.75 \pm 0.09 \text{ mM}$ on day 3 of cell culture, $n = 3$), which generated ROS to significant amounts. However, elevated glutathione levels were found in large, non-necrotic tumor spheroids with a maximum at day 12 of tumor spheroid cell culture ($11 \pm 0.85 \text{ mM}$, $n = 3$) corresponding to spheroid diameters of $\pm 250 \mu m$. This size class of tumor spheroids
roids consequently generated low levels of ROS (see Fig. 6A). Prolonged culture times (>12 days) resulted in a decline of the glutathione content, which is in line with the observed elevated ROS levels in large tumor spheroids containing extended central necrosis.

**Effects of Intracellular ROS Elevation on Pgp Expression in Multicellular Tumor Spheroids**—The working hypothesis of our present study is based on the assumption that the intracellular redox state of tumor spheroids regulates the expression of the MDR transporter Pgp. To address this issue, intracellular glutathione was reduced either by treatment of small tumor spheroids for 7 days, i.e., from day 5 to day 12 of cell culture, with 50 μM BSO, which is an irreversible inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme in glutathione biosynthesis (25) or by incubating tumor spheroids in glutamine-reduced cell culture medium (26). Both experimental conditions significantly increased ROS levels as compared with untreated control spheroids (Fig. 7) (n = 3) and significantly stimulated tumor spheroid growth (data not shown). The increased ROS levels could be completely inhibited by addition of ebselen, which is an effective free radical scavenger (27).

Elevation of intracellular ROS in large, non-necrotic multicellular tumor spheroids (diameter 100 ± 50 μm, days 3–6 of tumor spheroid culture) (filled circles), large non-necrotic tumor spheroids (diameter 300 ± 50 μm, days 10–15 of tumor spheroid culture) (filled squares), as well as large tumor spheroids containing central necrosis (diameter 500 ± 100 μm, days 18–40 of tumor spheroid culture) (filled triangles) were incubated with the ROS indicator H$_2$DCFDA, and the time course of the generation of fluorescent DCF was monitored. The data were fitted by linear regression. B, expression of the NADPH oxidase subunits p47$^{phox}$ and p67$^{phox}$ during cell culture of DU-145 tumor spheroids. The data were obtained by quantification of p47$^{phox}$ and p67$^{phox}$ immunofluorescence. C, evaluation of the glutathione content in DU-145 tumor spheroids after different times of cell culture. Note that the glutathione content is elevated in large non-necrotic tumor spheroids (days 7–12). The data were fitted by a Gaussian algorithm.
Redox-regulation of Pgp

Effects of decreased intracellular glutathione levels on the expression of Pgp (A) and doxorubicin retention (B) in large, non-necrotic tumor spheroids. Treatment of tumor spheroids for 7 days (days 5–12) either with 50 μM BSO or incubation in glutamine-reduced medium significantly reduced Pgp. The data represent the relative Pgp expression (%) on day 12 of cell culture in relation to the Pgp expression in tumor spheroids on day 5 of cell culture (see Fig. 1). The decrease in Pgp consequently resulted in augmented doxorubicin retention. The observed effects could be efficiently reversed by coadministration of the free radical scavenger ebselen. *, p < 0.05, significantly different from the untreated control.

Fig. 8. Effects of decreased intracellular glutathione levels on the expression of Pgp (A) and doxorubicin retention (B) in large, non-necrotic tumor spheroids. Treatment of tumor spheroids for 7 days (days 5–12) either with 50 μM BSO or incubation in glutamine-reduced medium significantly reduced Pgp. The data represent the relative Pgp expression (%) on day 12 of cell culture in relation to the Pgp expression in tumor spheroids on day 5 of cell culture (see Fig. 1). The decrease in Pgp consequently resulted in augmented doxorubicin retention. The observed effects could be efficiently reversed by coadministration of the free radical scavenger ebselen. *, p < 0.05, significantly different from the untreated control.

Redox-regulation of Pgp may be dose-dependent. To evaluate the effects of increasing ROS concentrations on the expression levels of Pgp, 6-day-old tumor spheroids displaying intermediate Pgp levels were incubated in cell culture media containing increasing concentrations of H2O2 ranging from 1 to 750 μM. The decrease in Pgp consequently resulted in augmented doxorubicin retention. The observed effects could be efficiently reversed by coadministration of the free radical scavenger ebselen. *, p < 0.05, significantly different from the untreated control.

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Pgp expression by BSO resulted from an activation of EGF-mediated signal transduction pathways (Fig. 11). To verify these assumptions large, non-necrotic tumor spheroids were either treated with tyrphostin AG 1478 (10 μM), which selectively inhibits EGF receptor kinase, or incubated for 24 h in serum-free medium and in serum-free medium supplemented with EGF in a concentration range of 2.5–50 ng/ml. The serum-free conditions were chosen to avoid a possible influence of EGF present in the fetal calf serum of the cell culture medium on the outcome of the experiments. Tyrphostin AG 1478 treatment as well as incubation in serum-free medium resulted in a significant up-regulation of Pgp as compared with control conditions. Upon treatment with 2.5, 5, 20, and 50 ng/ml EGF a dose-dependent down-regulation of Pgp to 70 ± 15%, 61 ± 19%, 34 ± 7%, and 55 ± 10%, respectively, was observed as compared with serum-free conditions (set to 100%) (n = 3) (Fig. 11A). Coadministration of 20 ng/ml EGF and the free radical scavenger ebselen (1 μM) totally abolished the down-regulation of Pgp observed by treatment with EGF alone, indicating that the down-regulation of Pgp was mediated via ROS. In a parallel set of experiments tumor spheroids were treated with BSO in the presence of specific antagonists of the EGF signaling pathway, i.e., the PKC inhibitors BIM-1 (1 μM) and Ro-31-8220 (0.5 μM), the Ras antagonist FPT inhibitor III (1 μM), the Raf inhibitor ZM 336372 (1 μM), and the MEK1 inhibitor PD98059 (20 μM), respectively (n = 3) (Fig. 11B). The ROS-mediated down-regulation of Pgp by BSO treatment was completely abolished upon inhibition of the Ras-mediated tyrosine kinase signaling pathway, which clearly indicates that EGF-mediated signal transduction pathways are negatively regulating Pgp expression. None of the applied antagonists of receptor tyrosine kinase pathways exerted significant effects on intracellular ROS levels (data not shown).

FIG. 9. Effect of increasing concentrations of H2O2 on the expression of Pgp (A) and cell lethality (B). Six-day-old tumor spheroids were treated for 24 h with either 1, 200, 500, or 750 μM H2O2. Pgp expression was evaluated by quantitative immunohistochemistry in whole mount tumor spheroids. Cell lethality was determined by analyzing lethal SYTOX green-positive cell nuclei. Note that Pgp expression was down-regulated upon treatment with non-toxic concentrations of H2O2, whereas under conditions of increased cell lethality an up-regulation of Pgp was observed. *, p < 0.05, significantly different from the untreated control.

DISCUSSION

The present study reports on the correlation of endogenous intracellular ROS levels and the intrinsic expression of Pgp in multicellular prostate tumor spheroids of different size classes. The transient expression of Pgp during the growth of tumor spheroids, and its correlation with cell quiescence is apparently a general feature of tumor tissues, because tumor spheroids cultivated from glioma, melanoma, and hepatoma tumor cells displayed comparable features of intrinsic expression of Pgp as observed in the DU-145 prostate cancer cell line. The observation of a transient increase of Pgp-mediated drug resistance during the growth of avascular micrometastases may be of clinical significance, because the outcome of chemotherapeutic treatment critically depends on the chemosensitivity of the treated neoplastic tissue, which may be predicted from levels of Pgp expression as well as the expression of cell cycle-related genes in tumor biopsies. In DU-145 tumor spheroids the intrinsic Pgp expression was clearly correlated to increased levels of the CDK inhibitors p27Kip1 and p21WAF-1, which are increased during cell cycle arrest. Consequently, tumor spheroids with elevated levels of Pgp expression displayed minor endogenous activity of the cell growth-associated MAPK-members ERK1,2 and JNK. There is increasing evidence that the growth of neoplastic tissues is controlled by the generation of ROS, which have been previously shown to arise in Ras-mediated signaling pathways (23). The data of the present study are in notion with these previous studies, because it is demonstrated that increased ROS generation was observed in small tumor spheroids that consist predominantly of exponentially growing Ki-67-positive cells as well as in large tumor spheroids containing...
Redox-regulation of Pgp

extended central necrosis. The increase in Ki-67 expression was paralleled by an augmented activity of JNK in small spheroids and large spheroids containing central necrosis as well as an increased activity of ERK1,2 in small tumor spheroids.

The correlation of elevated Pgp levels with reduced levels of ROS as well as diminished activity of ERK1,2 and JNK led us to hypothesize that raising intracellular ROS may down-regulate Pgp expression via up-regulation of ERK1,2 and JNK. An increase in intracellular ROS was achieved by: addition of hydrogen peroxide to the cell culture medium, depletion of intracellular glutathione by the use of BSO and glutamine-reduced cell culture medium, as well as treatment with EGF, which has been previously demonstrated to raise intracellular ROS (31). All these experimental procedures resulted in significant down-regulation of Pgp levels, indicating that ROS are involved in the signaling pathways that regulate the expression of Pgp. Interestingly, with hydrogen peroxide concentrations exceeding 500 μM an up-regulation of Pgp was observed. A rise in Pgp expression following treatment of cells with hydrogen peroxide has been previously demonstrated (15). In this study millimolar concentrations of H₂O₂ were administered, resulting in a parallel up-regulation of poly-(ADP-ribose) polymerase, which is a nuclear enzyme induced by DNA strand breaks. Furthermore, stress-induced expression of Pgp has been recently evidenced under various conditions, i.e., UV radiation (32), low external pH and osmotic shock (33) as well as heat stress (34). Most of these stress factors are accompanied by the generation of intracellular ROS. Under stress conditions the physiological role of Pgp may be related to the prevention of apoptosis (35), presumably by regulating caspase-dependent apoptotic pathways (36). This notion is furthermore supported by studies that demonstrated that overexpression of Pgp is correlated with increased levels of the anti-apoptotic Bcl-xL protein (37). ROS acting as signaling agents in the regulation of cell proliferation are in the nanomolar to micromolar range and are therefore significantly lower than the concentrations necessary to induce DNA strand breakage (38). Hence, intracellular ROS may exert differential effects on the regulation of MDR-related genes, which are dependent on their intracellular concentration.

In the present study raising intracellular ROS by depletion of glutathione resulted in a decline of p27Kip1 expression, whereas activity of ERK1,2 and JNK was significantly elevated, which indicates that in parallel to the down-regulation of Pgp expression a stimulation of the cell cycle was achieved, suggesting that cell cycle stimulation by ROS and the regulation of Pgp expression may share a common signal transduction pathway. This assumption was supported by experiments demonstrating that pharmacological inhibition of members of the receptor tyrosine kinase pathway utilized by EGF, and involving PKC and Ras/Raf as well as ERK1,2, abolished the observed down-regulation of Pgp following elevation of intracellular ROS. Interestingly, incubation of tumor spheroids in serum-free media and treatment with tyrphostin, which inhibits protein tyrosine kinases, including autophosphorylation of EGF receptor kinase, significantly increased the expression of Pgp in multicellular tumor spheroids. These findings support the notion that EGF present in the fetal calf serum used in the cell culture medium and/or EGF secreted by tumor cells may regulate Pgp expression via the activation of receptor tyrosine kinase signaling pathways.

The apparent effects of ROS on Pgp expression levels and cell cycle activity in multicellular tumor spheroids may be clinically utilized in anti-cancer trials. In EMT-6 tumor spheroids down-regulation of p27Kip1, which has been discussed as a regulator of MDR (39), by antisense nucleotides increased cell proliferation and sensitized tumor cells to 4-hydroperoxycyclophosphamide (39). A comparable chemosensitization in EMT-6 tumor spheroids was observed by treatment with hyaluronidase, which reduced intercellular communication and activated cell proliferation (40). Furthermore, stimulation of cell proliferation in noncycling hematopoietic progenitor cells and leukemic blasts resulted in a down-regulation of Pgp-mediated MDR (41). An elevation of ROS by depletion of intracellular glutathione sounds reasonable, because BSO has successfully been applied in vivo in cancer patients and was shown to increase the sensitivity of cancer cells toward antineoplastic drugs (42). Because most antineoplastic agents are acting against rapidly cycling cancer cells, the concomitant cell cycle stimulation and down-regulation of Pgp by ROS may offer new avenues for conventional chemotherapy. This may hold especially true for hormone-independent metastatic prostate tumors, which are characterized by a high fraction of cells resting in the G₀ phase of the cell cycle and a pronounced intrinsic resistance to a broad range of anti-cancer agents (9).

REFERENCES

1. Gottesman, M. M., and Pastan, I. (1993) Annu. Rev. Biochem. 62, 385–427
2. Simon, S. M., and Schindler, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3497–3504

FIG. 11. Regulation of Pgp expression in multicellular DU-145 tumor spheroids by receptor tyrosine kinase pathways. A, effects of tyrphostin AG 1478 (10 μM), which selectively inhibits EGF receptor kinase, serum-free conditions, and different concentrations of EGF on the expression of Pgp in large, non-necrotic tumor spheroids (diameter 300 ± 50 μm, day 14 of tumor spheroid culture). After 24 h of incubation the tumor spheroids were fixed and Pgp expression was quantified by immunohistochemistry. The data represent the relative Pgp immunofluorescence (%) in relation to the Pgp immunofluorescence obtained under serum-free conditions (set to 100%). Note, that the down-regulation of Pgp by EGF was totally abolished upon coadministration of the applied antagonists of receptor tyrosine kinase pathways. Note, that all applied antagonists of receptor tyrosine kinase pathways totally abolished the BSO-mediated down-regulation of Pgp. *, p < 0.05, significantly different from serum-free conditions. B, effects of antagonists of receptor tyrosine kinase pathways on the down-regulation of Pgp by BSO. Note, that all applied antagonists of receptor tyrosine kinase pathways totally abolished the BSO-mediated down-regulation of Pgp. *, p < 0.05, significantly different from the untreated control.

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3. Volm, M. (1998) Anticancer Res. 18, 2905–2917
4. Oda, Y., Schneider-Stock, R., Rys, J., Grzechala, A., Niezabitowski, A., and Roesser, A. (1996) Diagn. Mol. Pathol. 5, 98–106
5. Dhar, D. K., Nagasue, N., Yoshimura, H., Tachibana, M., Tahara, H., Matsuura, H., Abe, S., Chang, Y. C., and Nakamura, T. (1995) J. Surg. Oncol. 60, 50–54
6. Pilarski, L. M., and Belch, A. R. (1995) Leuk. Lymphoma 17, 367–374
7. Wartenberg, M., Frey, C., Diedershagen, H., Ritgen, J., Hescheler, J., and Sauer, H. (1998) Int. J. Cancer 73, 855–863
8. Wartenberg, M., Hescheler, J., Acker, H., Diedershagen, H., and Sauer, H. (1998) Cytometry 31, 137–145
9. Naito, S., Yokomizo, A., and Koga, H. (1999) Int. J. Urol. 6, 427–439
10. Beedassy, A., and Cardi, G. (1999) Semin. Oncol. 26, 428–438
11. Sarkadi, B., and Muller, M. (1997) Semin. Cancer Biol. 8, 171–182
12. Stein, U., Walther, W., Laurenzot, C. M., Scheffer, G. L., Scheper, R. J., and Shoemaker, R. H. (1997) J. Natl. Cancer Inst. 89, 807–813
13. Deshpande, S. S., Angkeow, P., Huang, J., Osaki, M., and Irani, K. (2000) FASEB J. 14, 1705–1714
14. Yamane, Y., Furuchi, M., Song, R., Van, N. T., Mulcahy, R. T., Ishikawa, T., and Kuo, M. T. (1998) J. Biol. Chem. 273, 31975–31985
15. Ziemann, C., Burkle, A., Kahl, G. F., and Hirsch-Ernst, K. I. (1999) Carcinogenesis 20, 407–414
16. Wartenberg, M., Fischer, K., Hescheler, J., and Sauer, H. (2000) Int. J. Cancer 88, 267–274
17. Frenkel, K., and Gleichauf, C. (1991) Free Radic. Res. Commun. 12–13, 783–784
18. Noeman, K. E., Beck, C., Holmzayer, T. A., Chin, E. J., Wunder, J. S., Andralis, I. L., Gazdar, A. F., Willman, C. L., Griffith, B., and Von Hoff, D. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7160–7164
19. Wartenberg, M., and Acker, H. (1996) Anticancer Res. 16, 573–579
20. Sauer, H., Hescheler, J., Reis, D., Diedershagen, H., Niedermeier, W., and Wartenberg, M. (1997) Br. J. Cancer 75, 1481–1488
21. Sztutowowicz, T. P., and Nathan, C. F. (1991) Cancer Res. 51, 794–798
22. Burdon, R. H. (1996) Biochem. Soc. Trans. 24, 1028–1032
23. Irani, K., Xia, Y., Zweier, J. L., Sollett, S. J., Der, C. J., Fearon, E. R., Sundaresan, M., Finkel, T., and Goldschmidt-Clermont, P. J. (1997) Science 275, 1649–1652
24. Wartenberg, M., Hescheler, J., and Sauer, H. (1997) Am. J. Physiol. 272, R1677–R1683
25. Griffith, O. W., and Meister, A. (1979) J. Biol. Chem. 254, 7558–7560
26. Dringen, R., and Hamprecht, B. (1996) J. Neurochem. 67, 1375–1382
27. Maizono, M., Roveri, A., and Ursini, F. (1992) Arch. Biochem. Biophys. 295, 404–409
28. Sauer, H., Diedershagen, H., Hescheler, J., and Wartenberg, M. (1997) FEBS Lett. 419, 201–205
29. Wartenberg, M., Diedershagen, H., Hescheler, J., and Sauer, H. (1999) J. Biol. Chem. 274, 27709–27717
30. Bae, Y. S., Kang, S. W., See, M. S., Eaines, I. C., Tekle, E., Clock, P. B., and Rhee, S. G. (1997) J. Biol. Chem. 272, 217–221
31. Uchiumi, T., Kohno, K., Tanimura, H., Matsuo, K., Sato, S., Uchida, Y., and Kuwano, M. (1993) Cell Growth & Differ. 4, 147–157
32. Wei, L. Y., and Roepe, P. D. (1994) Biochemistry 33, 7229–7238
33. Miyazaki, M., Kohno, K., Uchiumi, T., Tanimura, H., Matsuo, K., Nasu, M., and Kuwano, M. (1992) Biochem. Biophys. Res. Commun. 187, 677–684
34. Johnston, R. W., Creney, E., and Smyth, M. J. (1999) Blood 93, 1075–1085
35. Smyth, M. J., Kraanovskis, E., Sutton, V. R., and Johnston, R. W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7024–7029
36. Kohjima, H., Endo, K., Moriayama, H., Tanaka, Y., Almenri, E. S., Slapak, C. A., Teicher, H., Kufe, D., and Datta, R. (1998) J. Biol. Chem. 273, 16647–16650
37. Burdon, R. H., Gill, V., and Alliandangia, D. (1996) Free Radic. Res. 24, 81–93
38. St. Croix, B., Florenes, V. A., Rak, J. W., Flanagan, M., Bhattacharya, N., Slingerland, J. M., and Kerbel, R. S. (1996) Nat. Med. 2, 1204–1210
39. Croix, B. S., Rak, J. W., Kapitain, S., Sheehan, C., Graham, C. H., and Kerbel, R. S. (1996) J. Natl. Cancer Inst. 88, 1285–1296
40. Smeets, M. E., Raymakers, R. A., Vierwinden, G., Pennings, A. H., Wessels, H., and de Witte, T. (1999) Blood 94, 2414–2423
41. O'Dwyer, P. J., Hamilton, T. C., LaCreta, F. P., Gallo, J. M., Kilpatrick, D., Halbieri, T., Brennan, J., Bookman, M. A., Hoffman, J., Young, R. C., Comis, R. L., and Ozola, R. F. (1996) J. Clin. Oncol. 14, 249–256
Down-regulation of Intrinsic P-glycoprotein Expression in Multicellular Prostate Tumor Spheroids by Reactive Oxygen Species

Maria Wartenberg, Frederike C. Ling, Maurice Schallenberg, Anselm T. Bäumer, Kerstin Petrat, Jürgen Hescheler and Heinrich Sauer

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