Role of the tumor microenvironment in the activity and expression of the p-glycoprotein in human colon carcinoma cells

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Abstract. The metabolic microenvironment of solid tumors is characterized by an oxygen deficiency and increased anaerobic glycolysis leading to extracellular acidosis and ATP depletion, which in turn may affect other energy-dependent cellular pathways. Since many tumors overexpress active drug transporters (e.g. the p-glycoprotein) leading to a multidrug-resistant phenotype, this study analyzes the impact of the different aspects of the extracellular microenvironment (hypoxia and acidosis) on the activity and expression of the p-glycoprotein (pGP) in the human colon carcinoma cell line LS513. For up to 24 h cells were exposed to hypoxia (pO_2<0.5 mmHg), an acidic extracellular environment (pH 6.6), or the combination of hypoxia and acidosis. Under hypoxic conditions (at a normal pH), the pGP activity (measured by the daunorubicin efflux) and the pGP expression were not markedly altered. Under acidic conditions, however, the pGP-mediated drug efflux was increased, an effect which was even more pronounced when the cells were exposed to hypoxia and acidosis simultaneously (increasing the pGP-activity by 70%). The cellular pGP expression remained almost constant under these conditions, indicating that the increased transport rate results from a functional modulation. The findings of the present study indicate that the parameters of the tumor microenviroment (especially extracellular acidosis) can increase the pGP-mediated drug efflux, an effect which may explain the reduced cytotoxicity of chemotherapeutic agents in hypoxic/acidic tumors.

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Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+}-concentration; pGP, p-glycoprotein; DNR, daunorubicin; VPL, verapamil

Key words: p-glycoprotein, acidosis, hypoxia, drug efflux, intracellular pH

Introduction

Resistance to chemotherapeutic agents is a major factor affecting the long-term therapeutic outcome of tumor patients. One reason for the development of a chemoresistant phenotype is the expression of active transport mechanisms by which numerous drugs are pumped out of the cells and which may therefore be responsible for the chemoresistant phenotype (MDR, multidrug resistance) seen in a number of tumors. A large number of these transporters belonging to the ABC (ATP-binding cassette) family have been identified. The best-studied ABC-transporter is the p-glycoprotein (pGP) product of the MDR1 gene (1,2). This protein is able to actively pump various drugs (e.g. doxorubicin, vinblastine, and paclitaxel) out of the cell, thereby reducing the cytotoxic efficacy of these drugs (2,3). At the same time, various inhibitors of pGP have been found (e.g. verapamil and cyclosporine A), which are capable of reducing the chemoresistance of pGP-expressing tumor cells, at least in cell culture experiments (2,4,5).

In comparison to normal tissues, solid-growing tumors exhibit numerous structural and functional differences, in particular concerning the properties of the vascular network. The structural abnormalities include the blind endings of vessels, the loss of vascular hierarchy, increased vascular permeability, arterio-venous shunt perfusion (6,7), and may result in an inadequate perfusion which does not follow a regular pattern (7) thus leading to an insufficient O_2 supply to the tissue. As a consequence of these functional parameters, the metabolic microenvironment of a tumor is fundamentally different to that of normal tissues (7). The mean oxygen partial pressure (pO_2) in tumors is often considerably lower than in the surrounding normal tissue, with areas of severe hypoxia or even anoxia in the vital tumor tissue in ~60% of human tumors (8). Correspondingly, tumor cells switch to anaerobic glycolysis, resulting in high lactate and low glucose concentrations, pronounced extracellular acidosis with pH values in some instances even below 6.5, and a lack of ATP (7).

Since ABC-transporters use ATP hydrolysis as an energy source, the question arises of whether the adverse microenvironment (hypoxia and acidosis) affects the transport efficacy of pGP. The aim of this study therefore was to

analyze whether oxygen deficiency and/or the extracellular pH in tumors are capable of influencing the activity or expression of the p-glycoprotein.

Materials and methods

Materials. The pGP-antibody (clone C219) was purchased from Signet Laboratories (Dedham, MA, USA) and the secondary antibody (rabbit anti-mouse, HRP-conjugated) from BioTrend (Cologne, Germany). All the other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Cell line. The human colorectal carcinoma cell line LS513 (American Type Culture Collection, Rockville, MD, USA; CRL-2134) was used in all the experiments. This cell line is known to functionally express the p-glycoprotein. The cells were grown in RPMI medium supplemented with 10% fetal calf serum (FCS) at 37˚C under a humidified 5% CO_2 atmosphere and subcultured once a week. For the experiments, the cells grew either in culture flasks or in 96-well plates. Twenty-four hours prior to the experiments, the cells were transferred to the RPMI medium without FCS. For the control experiments, the medium was buffered with 20 mM HEPES adjusted to a pH of 7.5. For acidic conditions, the cells were incubated in a medium buffered with 20 mM MES (morpholinoethane-sulfonic acid) with 4.51 mM $NaHCO_3$, adjusted to a pH of 6.6. For hypoxic conditions, the medium was also buffered with HEPES adjusted to an initial pH at the beginning of the hypoxia incubation of 7.5. The cells were then exposed to a hypoxic atmosphere containing 95% N_2+5% CO_2 (pO_2<0.5 mmHg) for up to 24 h. For the combination of hypoxic and acidic conditions, the culture medium was buffered with MES and adjusted to pH 6.6 and the cells were simultaneously exposed to the hypoxic atmosphere (95% N_2+5% CO_2).

Daunorubicin uptake assay. In order to assess the activity of the p-glycoprotein, the uptake of daunorubicin (DNR) into the tumor cells was determined in the presence or absence of a specific pGP-inhibitor (verapamil) (9). For this, the cells were incubated with DNR at a final concentration of 4 μM (stock solution 0.1 mM, dissolved in isotonic NaCl solution) for 30 min at 37˚C. In a second set of experiments, the cells were incubated with a combination of DNR (4 μM) and verapamil (VPL) at a concentration of 10 μM (stock solution 5 mM, dissolved in EtOH). The intracellular DNR concentration after incubation was assessed by measuring the DNR-induced fluorescence by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 575 nm. In each experiment, 10^4 cells were analyzed. Since VPL inhibits the active efflux of DNR, the intracellular concentration is higher in the presence of verapamil. The DNR concentration ratio in the presence and absence of VPL ($FL_{DNR+VPL}/FL_{DNR}$) can therefore be used as a measure of pGP activity (9). All the pGP activity values measured following the incubation of the cells under hypoxic and/or acidic conditions were normalized with respect to the control values obtained under normoxia and normal cell culture conditions [$(FL_{DNR+VPL}/FL_{DNR})_{hypoxic}$/ $(FL_{DNR+VPL}/FL_{DNR})_{control}$ and $(FL_{DNR+VPL}/FL_{DNR})_{acidic}$/ $(FL_{DNR+VPL}/FL_{DNR})_{control}$].

pGP-expression. The cellular expression of pGP was determined in a whole cell ELISA as previously described (10). In brief, after cell fixation with 4% paraformaldehyde for 60 min, the cells were washed with a permeabilizing buffer containing 0.1% Triton X-100 and then incubated for 20 min with the same buffer to which 0.6% H_2O_2 was added. After incubation with the primary anti-pGP antibody (diluted 1:1000) at 4˚C overnight, the cells were washed and incubated with the secondary anti-mouse peroxidase antibody (diluted 1:1000) for 1 h. Finally, the cells were incubated with a HRP-substrate (containing 0.5 mg/ml o-phenylenediamine, 11.8 mg/ml Na_2HPO_4 x $2H_2O$, 7.3 mg/ml citric acid and 0.015% H_2O_2) for 15 min and measured photometrically at a wavelength of 490 nm using a microplate reader (Victor, Wallac, Turku, Finland). In order to normalize the pGP expression for the number of cells in each well, the permeabilized cells were subsequently incubated with 0.2% trypan blue solution for 5 min, washed with PBS, and dissolved with 1% SDS. The trypan blue concentration as a measure of the cell number was determined photometrically.

Intracellular pH measurement. The intracellular pH was determined using the pH-sensitive fluorescent dye 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) as previously described by Weiner and Hamm (11). For this, the cells were incubated on coverslips with BCECF (a final concentration of ~2 μM) for 5 min at 37˚C. Subsequently, the coverslips were transferred to an inverse microscope (Axiovert 100 TV, Zeiss, Oberkochen, Germany). The excitation wavelength was switched between 460 nm and 488 nm. Emission fluorescence was monitored at 535 nm using a CCD camera system (C4742-95, Hamamatsu Photonics, Herrsching, Germany) and image analysis software (Aquacosmos Ver. 1.3, Hamamatsu Photonics). After the subtraction of the background levels, the fluorescence intensity ratio at both excitation wavelengths was calculated every 10 sec. At the end of each measurement period, a pH calibration was performed. For this, two calibration solutions containing 132 mM KCI, 10 mM NaCl, 1 mM CaCl, 1 mM MgCl, 10 mM HEPES and 10 μM nigericin were prepared and adjusted to pH 6.8 and 7.6, respectively. The medium on the coverslips was replaced by these solutions and the fluorescence ratio was measured. The intracellular pH of each cell was calculated by the linear interpolation of the calibration points.

Metabolic parameters. The glucose and lactate concentrations of the medium were measured enzymatically using standard test kits (nos. 1447521 and 1822837; Roche-Diagnostics, Indianapolis, USA).

Statistical analysis. Results are expressed as the means ± standard error of the mean (SEM). The differences between the groups were assessed by the two-tailed Wilcoxon test for unpaired samples. The significance level was set at α=5% for all the comparisons.

Results

The expression of the functional p-glycoprotein by LS513 colon carcinoma cells was determined by the DNR uptake

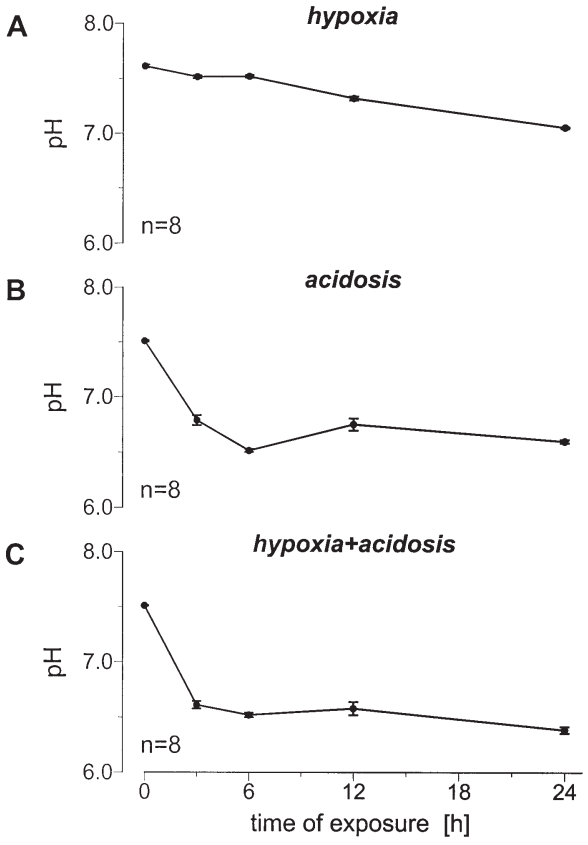

Figure 1. Changes in the extracellular pH in the medium under (A) hypoxic, (B) acidic, and (C) a combination of hypoxic and acidic conditions for up to 24 h. Values are expressed as the means ± SEM. All values were significantly ($p<0.002$) different from the pH at t=0 min. n, number of experiments.

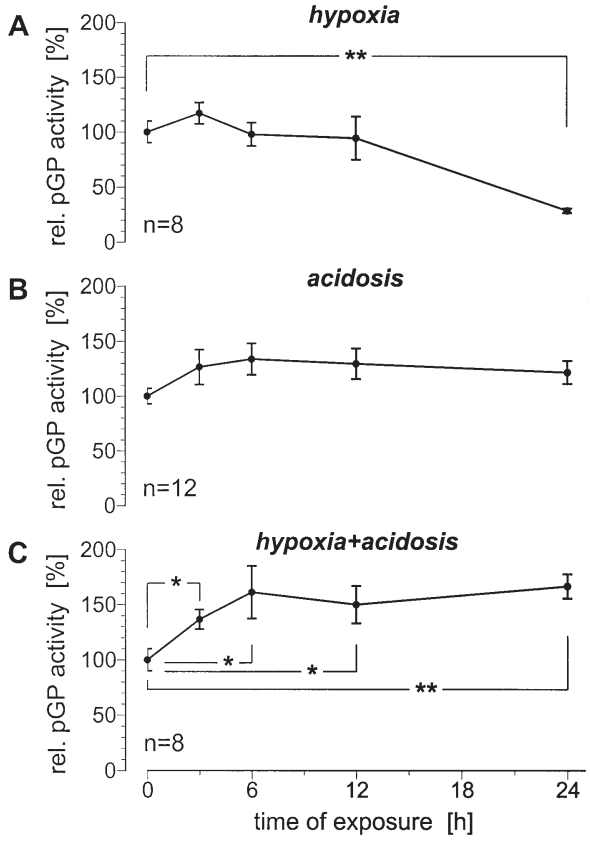

Figure 2. The relative activity of pGP (described by the ratio of the intracellular DNR concentration in the presence or absence of verapamil) in the cells under (A) hypoxic, (B) acidic, and (C) a combination of hypoxic and acidic conditions for up to 24 h. Values are expressed as the means ± SEM normalized to the activity under the control conditions at t=0 min. $^{*}p<0.05$; $^{**}p<0.01$; n, number of experiments.

assay. In the presence of the VPL inhibitor, the intracellular DNR concentration was higher by a factor of 3.15±0.18 compared to the cells incubated with DNR only (n=28, $p<0.001$). This ratio ($FL_{DNR+VPL}/FL_{DNR}$) was used as a measure of the pGP activity. Incubating the cells under various environmental conditions led to different changes in the functional activity of the pump (the pH values for the various experimental conditions, measured during the 24-h incubation are shown in Fig. 1). Under severe hypoxia ($pO_2<0.5$ mmHg) at a normal extracellular pH, the pGP activity remained almost constant for up to 12 h (Fig. 2A). After 24 h, the DNR efflux was reduced by ~50% as a result of cellular damage incurred by the long-lasting O_2-defficiency. After 24 h of hypoxic conditions, many cells had become detached from the Petri dish. A reduction of the extracellular pH to 6.6 (the pH in the medium is shown in Fig. 1B) led to an increase in the pGP activity of ~30% which endured over the whole 24-h observation period (Fig. 2B). This increase was, however, not statistically significant. In the third set of experiments where the cells were exposed to a combination of hypoxic and acidic conditions (the pH in the medium is shown in Fig. 1C), the pGP activity was significantly increased by 70% (Fig. 2C).

The higher pGP transport activity under the combined hypoxic and acidic conditions could be the result of either an increase in pGP expression or of a functional modulation of pre-existing transporters. Since the pGP expression was not markedly enhanced under any of the experimental conditions (hypoxic, acidic, or hypoxic and acidic; Fig. 3), the increase in the DNR uptake resulted from an increase in the functional activity of pGP or a translocation of the pre-existing transporters from the vesicles to the cell membrane.

In order to study the underlying mechanisms by which the extracellular environment can influence pGP activity, we measured the changes in the metabolic parameters and the intracellular pH. The maintenance of LS513 carcinoma cells under hypoxic conditions ($pO_2<0.5$ mmHg) for up to 24 h, forces the cells to switch to the anaerobic metabolic pathway as indicated by a reduction in the glucose concentration of down to 3 mM in the medium (Fig. 4A) and an increase in the lactate level of up to 4 mM (Fig. 5A). These changes in the metabolic parameters occurred in spite of the fact that the cells were incubated at a normal extracellular pH or additionally exposed to acidic conditions with a pH of 6.6 (Figs. 4C and 5C). When the cells were incubated in an acidic environment at a normal pO_2, the glucose concentration dropped only marginally (Fig. 4B) and the lactate level was significantly lower than in the hypoxic groups (Fig. 5B). The intracellular pH (pH_i) in the LS513 cells changed more or less in parallel to the extracellular pH (Fig. 6). The pH_i however, was always slightly lower (0.2 to 0.3 units less) than the extracellular pH in all the experimental groups. This difference between the intra-

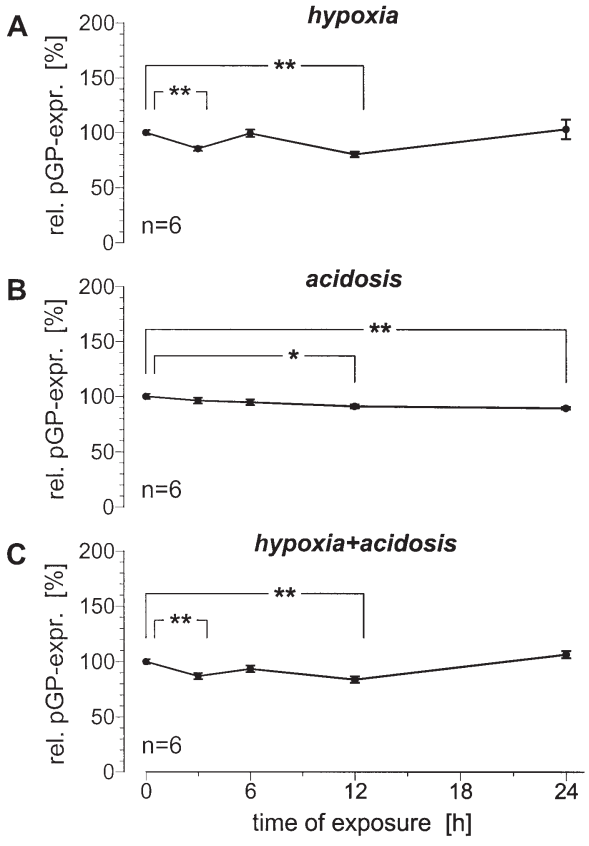

Figure 3. The relative pGP-expression of the cells under (A) hypoxic, (B) acidic, and (C) a combination of hypoxic and acidic conditions for up to 24 h. Values are expressed as the means ± SEM normalized to the expression under the control conditions at t=0 min. $^{*}p<0.05$, $^{**}p<0.01$; n, number of experiments.

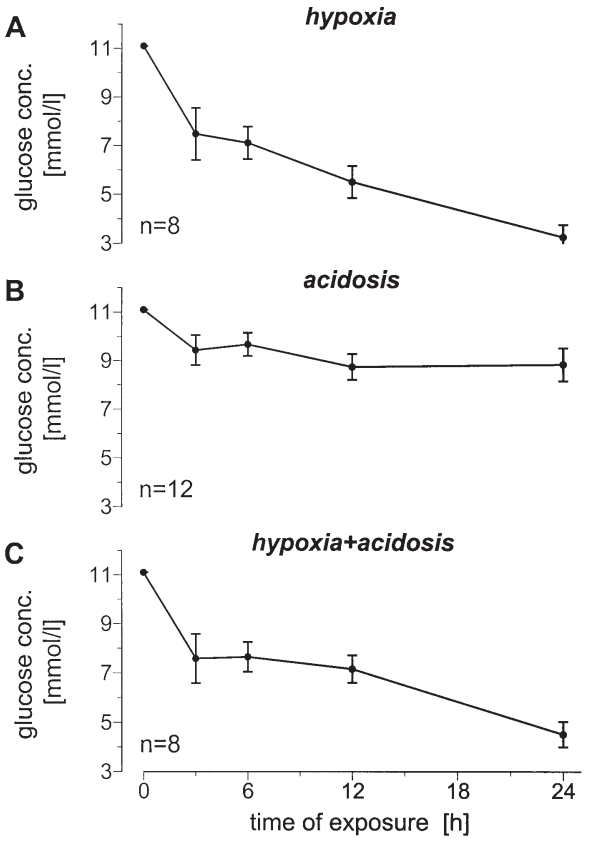

Figure 4. Changes in the glucose concentration in the medium under (A) hypoxic, (B) acidic, and (C) a combination of hypoxic and acidic conditions for up to 24 h. Values are expressed as the means ± SEM. All values were significantly ($p<0.01$) different from the concentration at t=0 min. n, number of experiments.

and extracellular pH was statistically significant in all the groups.

Discussion

The human colorectal carcinoma cell line LS513 functionally expresses the p-glycoprotein as indicated by the ~3x higher intracellular concentration of DNR (a known substrate of the pGP) (2,5) in the presence of the pGP-inhibitor verapamil. When using the DNR concentration ratio in the presence and absence of VPL as a measure of the functional pGP activity (9), the results of the present study indicate that the active drug efflux is modulated by the extracellular metabolic micro-environment. The insufficient oxygen supply to the tumor *in vivo* leads to pronounced tissue hypoxia/anoxia and thus commits the cells to glycolytic metabolism leading to extracellular acidosis with pH values sometimes even lower than 6.5 (7). Since the glycolytic pathway is less effective with respect to the ATP yield (7,12), it is conceivable that under hypoxic environmental conditions, the active (ATP-consuming) drug efflux can be reduced. However, even when the cells were maintained under anoxia for 12 h, no marked change was seen in the pGP activity (Fig. 2A). A reduction in the DNR efflux of ~50% was observed only after exposure to hypoxia for 24 h, thus reflecting cellular damage due to long-lasting anoxia. The measurement of metabolic parameters under these conditions (Figs. 4A and 5A) clearly indicates that even though the cells were committed to glycolysis (with lactate concentration increases of up to 4 mmol/l), the glucose level was nevertheless high enough (3.3±0.5 mmol/l) for the ATP supply to the cells to be maintained. It has, however, been proposed that the inhibition of glycolysis (leading to a dramatic decrease in the ATP level) could be a possible therapeutic strategy for overcoming drug resistance. Xu *et al* (13) showed that the inhibition of mitochondrial respiration leads to an increase in the cytotoxic efficacy of various chemo-therapeutic drugs as a result of a reduced pGP activity which was attributed to a depletion of ATP. However, in the present study, hypoxia for up to 12 h did not lead to a decrease in the ATP concentration sufficient enough to reduce the pGP activity. In addition, environmental hypoxia did not markedly change the pGP expression (Fig. 3A). These results are in accordance with previous studies showing that hypoxia/anoxia had almost no impact on the pGP expression (14,15). Only Comerford *et al* (16) found an induction of MDR1 after 48 h in an O_2-deprived medium. However, in the latter study, hypoxia was defined as a pO_2 in a culture medium of 20 mmHg which is not comparable to the pO_2 used in the present study and which is much higher than the pO_2 found in solid-growing tumors (7,8). The discrepancy between the studies may be due to the different cell lines used, divergent levels of hypoxia or differences in the duration of hypoxia exposure.

In contrast to the results obtained under hypoxic conditions, in the acidic environment (extracellular pH 6.6, Fig. 1), an

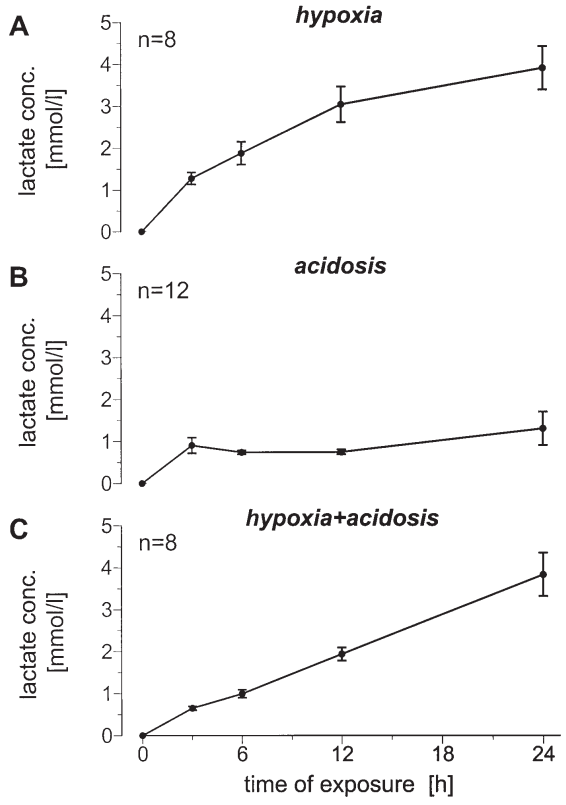

Figure 5. Changes in the lactate concentration in the medium under (A) hypoxic, (B) acidic, and (C) a combination of hypoxic and acidic conditions for up to 24 h. Values are expressed as the means ± SEM. All values were significantly ($p<0.01$) different to the concentration at t=0 min. n, number of experiments.

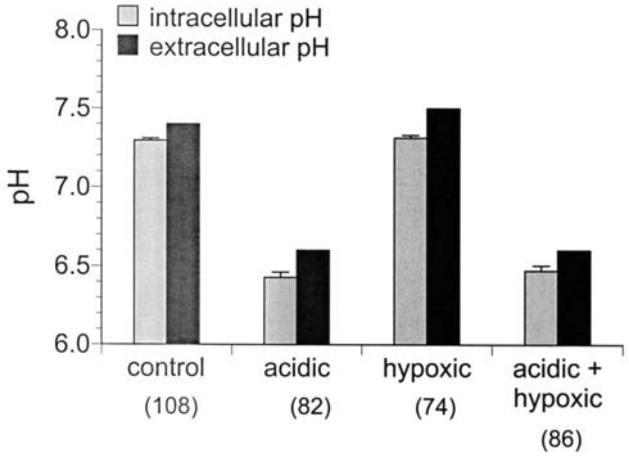

Figure 6. The intracellular pH of LS513-cells under the control, hypoxic, acidic, and hypoxic and acidic conditions (n, number of cells investigated). The extracellular pH values were significantly ($p<0.01$) higher than the intracellular pH in all the groups.

increased transport activity was seen which was most pronounced when the low pH was simultaneously combined with a reduced pO_2 (Fig. 2C). Under these conditions (a low pO_2 and pH), which is the common situation in human tumors (7), the DNR efflux increased significantly by 70%. Similar results have been found in a different cell line (rat R-3327-AT1 prostate carcinoma cells) measured with a rhodamine-123 efflux assay (17). Exposure of these cells to extracellular acidosis resulted in an increase in the pG activity by a factor of 2.4. In contrast to the LS513 cells used in the present study where the pGP-mediated efflux was elevated for up to 24 h, the AT1 cells showed only a transient increase in pump activity which lasted for <12 h. The higher transport rate seen in both studies upon cell incubation at a low extracellular pH could be due to (i) an increase in cellular pGP expression, (ii) a higher activity of the pre-existing transporters, or (iii) a translocation of pre-formed transporters to the outer cell membrane (which has already been suggested for pGP) (18,19). Even though the pGP expression has been shown to be increased by numerous environmental factors (such as glucose depletion, reactive oxygen species, hyperthermia or cytokines) (20-24), the acidic environment had almost no impact on the pGP expression (Fig. 3), indicating that the higher transport rate is predominantly the result of a functionally increased pump activity.

The question of how the extracellular pH may influence pGP activity has been addressed in a previous study (17). Besides cytokines (e.g. epidermal growth factor), the pGP phosphorylation by the protein kinase C (PKC) has also been discussed as a possible means of modulating the pGP activity at a posttranslational level (25,26). It has been shown that the activation of PKC by PMA can reduce the pGP-mediated drug efflux rate (27) and that the inhibition of PKC leads to a significant increase in the pGP-mediated drug efflux (17). However, the question of how the extracellular pH can influence the PKC activity has not yet been conclusively answered. In Fig. 6 it can be seen that the intracellular pH decreases within 5 to 10 min following a reduction in the extracellular pH. Such a pH-gradient has not been described *in vivo*. Several studies have demonstrated that the intracellular pH of solid-growing tumors remains almost constant even if the extracellular pH becomes considererably acidic (7). In cell culture experiments however, a decrease in the cellular pH, parallel to that of the medium pH and similar to that found in the present study has also been described (17,28,29). This discrepancy between *in vivo* and *in vitro* findings is unclear. It may be that the much larger extracellular space prevailing in the cell culture setting (as compared to solid tissues) in combination with the high buffer capacity of the cell culture medium represents a much larger H^+-pool leading to a pronounced H^+-influx into the cells which in turn cannot be compensated for by the cells' active proton pumps. In solid tissues, the extracellular H^+-pool is much smaller so that the cell is more likely to be capable of maintaining a constant intracellular pH. Further studies are necessary in order to answer the question of how the environmental pH affects the PKC activity (and by this the pGP-mediated drug efflux). For the modulation of PKC, the intracellular free Ca^{2+}-concentration ($[Ca^{2+}]_i$) plays an important role. Since several PKC isoforms are Ca^{2+}-dependent, one possibility could be a pH-dependent change in $[Ca^{2+}]_i$. In a previous study (17), a reduction in the intracellular Ca^{2+}-level of ~50% led to the pGP activity being more than doubled. The fact that low intracellular Ca^{2+}-levels have been reported for chemoresistant cell lines may also be an indication of the impact of Ca^{2+} in this context (30,31). It has been shown that extracellular acidosis leads to a very rapid (within 1 min) reduction in $[Ca^{2+}]_i$ which is much faster

than the change in the intracellular pH (17). From these results, there is likely to be a dependency of $[Ca^{2+}]_i$ on the extracellular pH, but not on the intracellular H^+-concentration (17). In order to conclusively answer the question of how the extracellular pH affects the intracellular Ca^{2+}-concentration and in turn influences the PKC and pGP activity, further *in vivo* experiments will be necessary. In particular, further elucidation is required into the question of how the combination of an acidic environment together with hypoxia increases the pGP activity (as compared to acidosis alone).

In conclusion, the present study clearly shows that hypoxia in the human colorectal carcinoma cell line LS513 has no marked impact on the activity or expression of pGP. In an acidic extracellular environment however, the functional activity is increased, an effect which is even more pronounced if the cells are exposed simultaneously to very low pO_2-values for several hours. These findings may be of significance for clinical oncology since they suggest that an alleviation of the extracellular acidity by supportive treatments can provide a means of increasing the chemosensitivity of tumor cells.

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