Hyperoxia-induced Improvement of the In Vitro Response to Gemcitabine in Transitional Cell Carcinoma

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Abstract. Background: Anemic tissue hypoxia can decrease the effects of chemotherapy in bladder cancer. Hypoxia leads to overexpression of hypoxia-inducible factor (HIF) and increased synthesis of epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). Tumor cell survival, invasion and angiogenesis thereby compromise treatment. Materials and Methods: Monolayer cultures of RT112, RT4, T24 and TCC SUP cells were incubated with or without gemcitabine and different gas mixtures (hypoxia, normoxia or hyperoxia). Cell proliferation (microculture tetrazolium assay), VEGF (enzyme-linked immunosorbent assay) and HIF-1α (Western blot analysis) were determined. Results: Hypoxia led to increased proliferation of transitional cell carcinoma (TCC) cells and elevated levels of HIF-1α and VEGF. Hyperoxia inhibited cell growth and lowered the concentration of VEGF. Treatment with gemcitabine was less effective under hypoxia. Conclusion: Hypoxia enhances TCC growth and may intensify angiogenesis mediated by VEGF. Hypoxia compromises treatment with gemcitabine. Correction of anemia might provide advantages in chemotherapeutic strategies for TCC.

Despite the ongoing development of new chemotherapeutic agents, the outcome of metastatic bladder cancer is still poor. However, new biological approaches to tumor cell death might redefine the role of established chemotherapeutic agents. Changes in the tumor’s microenvironment can lead to alterations in tumor cell biology or cell cycle. Therefore, interactions with chemotherapeutic agents might occur. These effects are partially known and are widely utilized in the radiotherapy of various tumors; for example, pre-oxygenation can enhance the cell damage caused by irradiation (1). Head and neck tumors in particular are treated according to these strategies. Additionally, a direct correlation between tumor oxygenation and blood hemoglobin concentration has been found. A comparison of anemic and non-anemic mice, which underwent radiotherapy for a subcutaneously-implanted sarcoma, revealed a significantly enhanced tumor shrinkage in the non-anemic animals (2).

The impact of tumor-associated hypoxia on chemotherapy has not yet been completely resolved. The cytotoxicity of carboplatin, adriamycin and paclitaxel is enhanced in the presence of oxygen (3, 4). Animal models demonstrated these effects especially in breast cancer cells (5, 6). Tumor-associated hypoxia induces overexpression of HIF-1α (hypoxia-inducible factor 1α). Thus, the syntheses of VEGF (vascular endothelial growth factor), EGF (epidermal growth factor) and IGF-2 (insulin-like growth factor 2) are up-regulated (7). To date, no data regarding chemotherapy for bladder cancer and hypoxia are available. As a consequence, an in vitro model has been established to investigate the effects of chemotherapy under different oxygen conditions in various human transitional cell carcinoma (TCC) cell lines. Gemcitabine (in combination with cisplatin) plays a substantial role in chemotherapy for bladder cancer. Moreover, a recent multicenter study suggests this combination as a reasonable alternative to the MVAC (methotrexate / vinblastine / doxorubicin / cisplatin) regimen (8). Thus, alteration of gemcitabine effects under hypoxia and hyperoxia has become an essential part of our study.

Materials and Methods

Tumor cell lines and culture conditions. Human transitional cell carcinoma cell lines RT112 (G1, p53 wild-type), RT4 (G1-2, p53 wild-type), T24 (G3, p53 mutant-type) and SUP (G4, p53 mutant-type) were obtained from ATCC (Rockville, MD, USA). All tumor cell lines were maintained as adherent cells at 37°C in an humidified atmosphere in RPMI 1640 culture medium (Biochrom, Berlin, Germany) supplemented with 10% heat-

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inactivated fetal calf serum (FCS), 1% non-essential amino acids, 2 mM L-glutamine, 100 units/ml penicillin G, 100 µg/ml streptomycin and vitamin solution (Gibco BRL, Life Technologies Inc., Frederick, MD, USA). For experiments, 1x10^6 tumor cells were seeded in 25-cm² culture flasks (Costar, Cambridge) and incubated for 48 h to allow maximum confluence, thus providing optimal cell-cell contact. The cells were routinely subcultivated by trypsinization (0.05% trypsin, 1 mM EDTA, Gibco BRL) and were incubated in medium with or without gemcitabine (10, 50, 250 nM) under different O₂ conditions (1% O₂ + 5% CO₂ + 94% N₂ (hypoxia), 21% O₂ + 79% N₂ (normoxia) or 95% O₂ + 5% CO₂ (hyperoxia)). These gemcitabine concentrations had been previously tested in vitro for their dose-effect relationship. A polarographic LICOX-probe (Integra Neurosciences, Hampshire) was used for oxygen tension monitoring in the culture medium.

Measurement of cell viability and proliferation by the microculture tetrazolium assay. The EZ4U (Microculture Tetrazolium) assay was used to measure cell viability and proliferation. Trypsinized tumor cells were resuspended in medium at 5x10^6 cells/ml after cell viability had been verified by trypan blue exclusion. One hundred µl of cell suspension was distributed into each well of a 96-well flat-bottomed microtiter plate and each plate was incubated to allow adherent cell growth. Following the incubation, the medium was removed and 200 µl of the different reagent solutions in complete medium (without Phenol red), or medium alone as a control, were distributed into each well. A working solution (Biozol, Eching, Germany) was added and further incubated for 4 h. The absorbance (A) values of each well were read at 450 nm using an automatic multiwell spectrophotometer (Spectra SLT, Crailsheim, Germany). The negative control (i.e., medium without cells), was used as the base-line absorbance. Statistical analysis was performed with the log-rank test.

Measurement of VEGF – protein concentration by enzyme-linked immunosorbent assay (ELISA). The assay set up was similar to that of the cell viability assay except that 2x10^5 cells were cultivated in 24-well plates with 1 ml medium. Cells were trypsinized, counted and diluted in medium at 2x10^6 viable cells/ml. One ml of the cell suspension was plated into each well of a 24-well flat-bottomed microtiter plate. Each plate was incubated overnight to allow cell attachment. The medium was replaced by 1 ml of the different reagent solutions in complete medium or medium alone as a control. After incubation for 24, 48 and 72 h under different O₂ conditions, cell culture supernatants were collected, particles removed by centrifugation and samples stored at ~20°C.

The Quantikine human VEGF Immunoassay (R & D Systems, Wiesbaden, Germany) employs the quantitative sandwich-enzyme immunoassay technique. A monoclonal antibody specific for VEGF is pre-coated onto the microplate. Standards and samples are pipetted into the wells and any VEGF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF is added to the wells. Following a further wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of VEGF bound in the initial step. The color development is then stopped and the color intensity measured.

Measurement of HIF-1α by Western blot analysis. 1x10^6 tumor cells were seeded in 25-cm² culture flasks and incubated overnight to allow for attachment. The medium was replaced by 5 ml of fresh medium, with or without supplements (control). The cells were treated for 24, 48 and 72 h under different O₂ atmospheres. After centrifugation, the protein concentration of the supernatant was determined with the BIO-RAD Protein Assay, a Bradford method (BIO-RAD Laboratories, München, Germany). Four hundred µl of the supernatant and 100 µl 5x Laemmli buffer (0.312 M Tris pH 6.8, 10% mercaptoethanol, 25% glycerol, 0.5% bromphenol blue, 10% SDS) were boiled for 5 min at 95°C for denaturation. Each sample was loaded with 20 µg protein/lane on a 10% SDS gel and separated. After transfer onto a nitrocellulose membrane, an antibody staining procedure was performed with a monoclonal anti-HIF-1α-MiG (10 µg/ml solution, Pharmingen, Hamburg, Germany). A goat anti-mouse IgG peroxidase conjugated antibody (1:10000 diluted, BIO-RAD Laboratories) was used as a secondary detection reagent.

Results

Measurement of cell viability and proliferation by the microculture tetrazolium assay. Under hypoxic conditions, cell viability and proliferation were seen to increase in some cell lines, as shown in Figure 1. Hypoxia led to a growth inhibition compared to hypoxic and normoxic conditions in the RT112 and TCC Supp cell lines. Therapy with gemcitabine produced a dose-dependent cell death in all cell lines (data not shown), but this effect was significantly weakened by hyperoxia and inhibited by hypoxia. In Figure 2, a gemcitabine dosage of 50 nM is shown.

Measurement of VEGF protein by enzyme-linked immunosorbent assay (ELISA). Hypoxia led to an increased expression of VEGF in all cell lines, as outlined in Figure 3. This effect increased with exposure time and seemed to be greater in the poorly-differentiated cell lines T24 and TCC Supp.

Measurement of HIF-1α by Western blot analysis. HIF-1α was up-regulated in all cells during hypoxia. Differences in up-regulation could be observed especially between the RT112 and T24 cell lines, as shown in Figure 4. TCC supp and RT4 cells are not shown.

Discussion

The aim of this study was to elucidate the impact of hypoxia on tumor cell proliferation and mediators of angiogenesis in TCC. Hyperoxia had a growth-inhibiting effect in TCC and, accordingly, hypoxia led to enhanced cell proliferation. This effect was seen in all cell lines, independent of grading or p53 status. These observations seem to be contradictory to those made in the studies of Xia et al. and Turner et al. (9, 10), who found differences in superficial and invasive bladder cancer regarding the expression of HIF. The results presented here,
however, confirm the idea of hypoxia as an important factor in tumor progression. HIF leads to an increased synthesis of VEGF and EGF, which might convert a superficial tumor into an invasive and metastasizing bladder cancer. Additionally, as a possible explanation for inhibiting tumor growth by hyperoxia, reduced cellular levels of EGF were found to have an impact on Fas-induced apoptosis, since EGF mediates protection against apoptosis by oxidizing intracellular levels of reduced glutathione (11). However, it has to be stated that the extreme O2 conditions, as chosen in

Figure 1. Cell proliferation (EZ4U) under different O2 conditions (normoxia= 21% O2 + 79% N2, hyperoxia: 95% O2 + 5% CO2, hypoxia 1% O2 + 5% CO2 + 94% N2). Each symbol represents the mean of 6 measurements. In statistical tests, p<0.05 is considered as significant compared to 'normoxia' as reference values.
our in vitro experiments, can hardly be transferred to a complex microenvironment like a tumor cell mass in vivo. As a further observation, hypoxia weakens the cytotoxic effects of gemcitabine, especially in poorly-differentiated cell lines. These effects have not been observed previously and the cellular interactions involved are not exactly known.

Gemcitabine requires phosphorylation to mono-, di- and triphosphates to be active. The first crucial step in phosphorylation is catalyzed by deoxycytidine kinase (dCK) and, therefore, an adequate pool of phosphorylated adenosine from mitochondrial O₂-dependent synthesis is essential (12). Here, hyperoxia and, accordingly, hypoxia...
might influence the effectiveness of gemcitabine. On the other hand, it seems most likely that hyperoxia shifts the relationship of cell proliferation and stagnation. Through this, the "target-cell population" for gemcitabine changes. As a result, hyperoxia might "pseudo-weaken" gemcitabine effects. Additionally, it remains unclear if hyperoxia-induced cell death is an issue in transitional cells. High levels of oxygen are known to be neurotoxic (13) and might also damage alveolar and retinal cells in vivo (14, 15).

As in vitro data are not easily transferable to the clinical setting, an in vivo model should be established to confirm the adverse effect of hypoxia on tumor cell growth in TCC. Data
on chemotherapy with gemcitabine during hyperoxia are promising and warrant further investigation in in vivo studies.

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References