

Influence of chronic hypoxia and radiation quality on cell survival

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To investigate the influence of chronic hypoxia and anoxia on cell survival after low- and high-LET radiation, CHO-K1 cells were kept for 24 h under chronic hypoxia (94.5% N₂; 5% CO₂; 0.5% O₂) or chronic anoxia (95% N₂; 5% CO₂). Irradiation was performed using 250 kVp X-rays or carbon ions with a dose average LET of 100 keV/μm either directly under the chronic oxygenation states, or at different time points after reoxygenation. Moreover, the cell cycle distribution for cells irradiated under different chronic oxidic states was measured over 24 h during reoxygenation. The measurements showed a fairly uniform cell cycle distribution under chronic hypoxia, similar to normoxic conditions. Chronic anoxia induced a block in G1 and a strong reduction of S-phase cells. A distribution similar to normoxic conditions was reached after 12 h of reoxygenation. CHO cells had a similar survival under both acute and chronic hypoxia. In contrast, survival after irradiation under chronic anoxia was slightly reduced compared to that under acute anoxia. We conclude that, in hamster cells, chronic anoxia is less effective than acute anoxia in inducing radioresistance for both X-rays and carbon ions, whereas in hypoxia, acute and chronic exposures have a similar impact on cell killing.

Keywords: radiosensitivity; hypoxia; anoxia; carbon ions; cell cycle distribution

INTRODUCTION

Hypoxia is known as a major reason for the resistance of tumor cells to radiation, and also to some O₂-dependent cytotoxic agents, by directly depriving cells of oxygen. Hypoxia also ultimately leads to an enhanced locoregional spreading of malignant cells and enhances the risk of metastatic formations. For more than 50 years it has been known that the response of cells to ionizing radiation is strongly dependent upon oxygen concentration [1, 2].

Acute hypoxia is caused by a temporary occlusion of a tumor blood vessel. Regions of such transient hypoxia in the tumor can vary within a short time. Chronic hypoxia is induced by insufficient oxygen supply due to the increasing

distance of cells in a growing tumor from the blood vessels, slowing reoxygenation. The oxygen gradient in such a tumor will normally range from oxidic to complete anoxia, i.e. to a necrotic level, in around 150 μm [3]. The range of hypoxia in which cells can still proliferate, thus increasing the tumor radioresistance, depends on the histology of the tissue [4].

Acute hypoxia has no direct influence on the metabolic status of the cells, whereas chronic hypoxia induces significant changes [5]. These changes may also influence the radiosensitivity. Some researchers have found that chronic hypoxia reduces radioresistance compared to acute hypoxia [6, 7]; others contend that chronic and acute hypoxia similarly modulate radiosensitivity [8]. Besides the increased

radioresistance, a direct result of oxygen deprivation, hypoxia has also been shown to affect cell cycle kinetics [9, 10].

During recent decades, several basic strategies have been considered for overcoming tumor hypoxia, and these have had promising results in some tumors in clinical trials, yet hypoxia-specific strategies are often disappointing because of the imbalance between efficiency and safety in unselected patient cohorts [11, 12]. Having an extremely precise distribution of physical doses and enhanced relative biological effectiveness (RBE), heavy ion beams are highly attractive for treating resistant or otherwise non-operable malignant tumors [13–15]. In particular, several studies suggest that the effectiveness of high linear energy transfer (LET) radiation on tumor-cell killing is less dependent on oxygen concentration than low-LET [15–18].

We studied the effect of chronic hypoxia on radiosensitivity and on cell cycle distribution to explore the role of LET on this influence.

MATERIALS AND METHODS

Hypoxic conditions and irradiation

For these experiments, a specially designed hypoxia exposure chamber was used, which can simulate different oxygenation situations with cell cultures and which allows for irradiation under hypoxic or anoxic conditions with X-rays as well as with ion beams. The construction and exact function of the chamber is described elsewhere (W. Tinganelli *et al.*, submitted for publication). Briefly, cells were grown on a gas-permeable foil in special designed rings. The rings were closed, transferred into the hypoxic chamber and gassed for 2 h with 95% nitrogen and 5% CO₂ to reach anoxic conditions, or with 94.5% nitrogen, 0.5% oxygen and 5% CO₂ to reach hypoxic conditions. To induce chronic hypoxia or anoxia afterwards, cells were kept for 24 h under hypoxic or anoxic conditions inside the chambers in the incubator. For measurements of the influence of reoxygenation, the rings were exposed to air again for the specified time.

Low-LET irradiation was carried out with an X-ray 250 kVp tube at 16 mA. Carbon irradiation was performed at the GSI synchrotron (SIS18) using a 1-cm spread-out Bragg-peak (SOBP) at a dose-averaged linear energy transfer LET_D = 100 keV/μm. Survival measurements of X-ray irradiation were usually performed 3–5 times, while those of carbon ions were only performed twice.

Cell culture and sample processing

We irradiated CHO-K1 (Chinese hamster ovary) cells, originally obtained from ATCC (No. CCL 61). Cells were cultivated in Ham's F12 medium supplemented with 10% fetal calf serum (FCS), and 1% Penicillin/Streptomycin (all Biochrom AG, Berlin, Germany) and maintained in a

humidified atmosphere of 5% CO₂ at 37°C. The cells were routinely checked for mycoplasma contamination and subcultured twice a week with 5 × 10⁴ cells in 25-cm² culture flasks with 5 ml culture medium.

For these experiments, 5 × 10⁴ cells were plated into specially designed rings and incubated for 24 h prior to gassing. After irradiation, cells were plated for a colony-forming assay according to standard procedures.

Cell data were fitted using the least-squares method by the linear-quadratic equation for X-ray irradiation and by a linear equation for carbon ion irradiation. Oxygen enhancement ratio (OER) and RBE values were calculated according to the usual formulas:

$$\text{OER}_S = \text{dose}_{\text{hypoxic}} / \text{dose}_{\text{oxic}}, \text{ and}$$

$$\text{RBE}_S = \text{dose}_{\text{x-ray}} / \text{dose}_{\text{ions}},$$

where S represents the survival level.

Realtime imaging of hypoxic cells

To observe directly the effect of the aerobic state on proliferation, a special chamber had to be designed that allowed us to keep the cells under controlled oxygen conditions while under the microscope. This chamber was adapted to an inverse phase-contrast microscope (Leica DM IRBE) to improve the image quality. For these experiments, 1000 CHO-K1 cells were seeded into ø 35 mm Petri dishes and kept in a humidified atmosphere at 37° with 5% CO₂. After 24 h the samples were transferred to the chamber and kept either under aerobic or hypoxic conditions. The chamber was placed on a remote-controlled microscope stage and the relevant positions in the cultivation vessels were first manually selected and stored. Then, during 24 h the microscope automatically took pictures from these positions. The number of cells was counted for each time point, and the local doubling time calculated using at least 10 areas per dish on three independent dishes. Results from different areas were statistically compatible with a Gaussian distribution, and data were therefore pooled to calculate the mean and standard error.

Fluorescent Activated Cell Sorting

A standardized flow cytometry protocol was used to measure the cell cycle fractions of CHO-K1 cells. Around 1–5 × 10⁵ cells were washed with PBS, fixed with ice cold 70% (v/v) ethanol and stored at –20°C for a maximum of 4 weeks. Before measuring, the ethanol inside the samples was removed, and the cells were stained for a minimum of 30 min with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) solution at room temperature while protected from light. The samples were measured with a PASIII flow cytometer (Partec, Münster, Germany) and analyzed with the Flowjo Program (Tree Star Inc., Ashland, USA).

RESULTS

Effects on clonogenic survival

Cell proliferation

To investigate the influence of the oxygenation state on cell proliferation, cells were kept for various durations under hypoxic or anoxic conditions. Afterwards, they were plated for a colony-forming assay. Growth under hypoxia did not differ significantly from aerobic conditions, resulting in an average plating efficiency (PE) of 0.80 ± 0.09 after 2–30 h of chronic hypoxia, compared to 0.79 ± 0.06 under oxic conditions. Real-time imaging measurements also showed no essential influence of hypoxia on the proliferation behavior of CHO-K1 cells. Data are shown in Table 1.

In contrast, cell proliferation was nearly stopped under anoxia. With real-time imaging, the doubling time for the totally anoxic cells exceeded the measurement time and could not be determined. After 2–30 h of anoxia, the plating efficiency showed a constant decrease to around 50% of the initial PE, indicating that only a certain subgroup of the cells recovered.

OER after X-ray irradiation

In Fig. 1, the clonogenic survival of CHO-K1 cells after X-ray irradiation for the chronic states of oxygenation is shown and compared to data under acute oxygenation

Table 1. Doubling times of CHO-K1 cells under oxic or hypoxia

oxic state	local doubling time (h)
oxic	11.5 ± 1.0
hypoxic	12.6 ± 3.4

conditions from (W. Tinganelli *et al.*, submitted for publication). All the experiments described here and in (W. Tinganelli *et al.*, submitted for publication) were performed simultaneously. Cell survival under chronic anoxia is significantly reduced compared to acute anoxia, but there is no statistically relevant difference between the survival curves under acute and chronic hypoxia. In Table 2, the alpha and beta values of a linear–quadratic fit are shown for these curves.

In consequence of their decreased survival, the resulting OER values under chronic anoxia are decreased compared to acute anoxia. In both cases, the OER after X-ray irradiation depends on survival level, but this effect seem to be less expressed under chronic anoxia. For chronic and acute hypoxia the OER values are similar. In Table 3 the OER values are shown for a survival level of 50, 10 and 1%.

OER and RBE after carbon irradiation

In addition to the X-ray experiments, we measured survival after C-ion irradiation under chronic oxygenation states. For irradiation with carbon ions, a 1-cm extended Bragg peak with a dose-averaged $LET_D = 100$ keV/ μ m was used. Survival curves after irradiation under chronic anoxia and hypoxia are shown in Fig. 2 and compared to data from acute anoxia and hypoxia from (W. Tinganelli *et al.*, submitted for publication), where experiments were performed simultaneously. Survival curves at this LET value can be regarded as exponential and fitted with a linear polynomial. Survival after carbon ion irradiation under chronic anoxia compared to acute anoxia is reduced in the same range as after X-ray irradiation. Survival under chronic and acute hypoxia is also similar after carbon irradiation. The resulting RBE and OER values are shown in Table 3. As the curves after carbon irradiation are exponential for oxic,

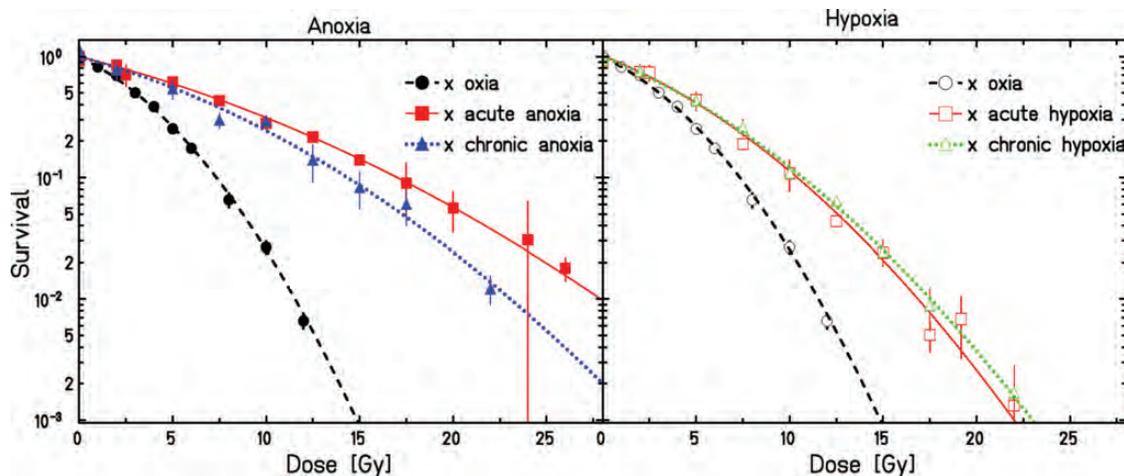


Fig. 1. Survival of CHO-K1 cells after X-ray irradiation under different states of oxygenation. Data for acute states are from (W. Tinganelli *et al.*, submitted for publication) (simultaneous experiments).

hypoxic and anoxic survival, the OER does not depend on survival level.

Effects of reoxygenation

To investigate the effect of reoxygenation on survival the cells were kept for 24 h under chronic anoxia or hypoxia, then the chambers were opened and the cells were kept for various durations in the incubator under normoxic conditions. Measurement of the oxygen concentration with an Oxygen Microsensor (NTH) showed that an oxygen concentration $>3\%$ was reached within 8–10 min after chronic anoxia and 7–9 minutes after chronic hypoxia, respectively. Full normoxic concentration of around 21% was reached after 60 min of reoxygenation.

Exposure to X-rays of formerly chronic anoxic cells was performed after 0 h (medium change for fast reoxygenation), 1 and 15 h of reoxygenation. Additionally, irradiation with carbon ions was performed after 1 h of reoxygenation. Formerly chronic hypoxic cells were X-ray irradiated after 1 and 24 h of reoxygenation. Furthermore, cells that had been incubated for 72 h under chronic hypoxia were irradiated after 1 h of reoxygenation. The results are shown in

Table 2. Alpha and beta values of CHO-K1 cells after X-ray irradiation under different states of oxygenation

	oxic state	$\alpha[\text{Gy}^{-1}]$	$\beta[\text{Gy}^{-2}]$
acute	oxic	0.164 ± 0.005	0.020 ± 0.001
	hypoxic	0.140 ± 0.017	0.0079 ± 0.001
	anoxic	0.089 ± 0.01	0.0027 ± 0.0005
chronic	hypoxic	0.141 ± 0.014	0.0070 ± 0.001
	anoxic	0.095 ± 0.023	0.0045 ± 0.001

Data for acute states are from (W. Tinganelli *et al.*, submitted for publication).

Table 3. OER and RBE values of CHO-K1 cells after X-ray and carbon ion irradiation under different states of oxygenation

	oxic state	RBE ₅₀	RBE ₁₀	RBE ₁	OER ₅₀	OER ₁₀	OER ₁
X-ray-acute	oxic	–	–	–	–	–	–
	hypoxic	–	–	–	1.31 ± 0.04	1.4 ± 0.04	1.45 ± 0.04
	anoxic	–	–	–	2.12 ± 0.07	2.31 ± 0.08	2.41 ± 0.08
X-ray-chronic	hypoxic	–	–	–	1.36 ± 0.24	1.46 ± 0.10	1.51 ± 0.11
	anoxic	–	–	–	1.90 ± 0.39	1.97 ± 0.16	2.00 ± 0.18
C-100-acute	oxic	3.59 ± 0.11	2.60 ± 0.07	2.04 ± 0.06	–	–	–
	hypoxic	3.73 ± 0.27	2.88 ± 0.21	2.33 ± 0.17	–	1.27 ± 0.09	–
	anoxic	3.84 ± 0.24	3.03 ± 0.19	2.45 ± 0.15	–	1.98 ± 0.12	–
C-100-chronic	hypoxic	3.71 ± 0.53	2.92 ± 0.26	2.39 ± 0.20	–	1.31 ± 0.12	–
	anoxic	4.06 ± 0.77	3.07 ± 0.35	2.46 ± 0.28	–	1.68 ± 0.18	–

Data for acute states are from (W. Tinganelli *et al.*, submitted for publication).

Fig. 3 and compared to measurements under normoxic conditions from (W. Tinganelli *et al.*, submitted for publication).

After chronic anoxia the survival after 0 and 1 h reoxygenation is reduced compared to normoxia. After 15 h of reoxygenation the enhanced sensibility has disappeared. The slightly higher survival, compared to cells irradiated under normoxia, may be due to the fact that only a subgroup survived 24 h of anoxia. Cells irradiated with carbon ions after 1 h of reoxygenation showed a similar increase in radiosensitivity. Chronic hypoxia does not affect the re-oxic sensibility of CHO-K1 cells even after longer treatment periods, as can be seen after the hypoxic treatment of 72 h.

Effects on cell cycle distribution

The cell cycle distribution was measured after 2–30 h of chronic anoxia and hypoxia to investigate the correlation between anoxia-induced changes in cell cycle and OER. Hypoxia did not significantly change the cell cycle distribution compared to cells grown under oxic conditions.

During the first 6 h the cell cycle distribution did not differ significantly from that under normoxic conditions. Afterwards, chronic anoxia led to an accumulation of cells in the G1 phase and a marked decrease in cells in S-phase. After 20 h a stable state was reached. Therefore, 0 and 24 h after gassing, were chosen as acute and chronic states. In Fig. 4 the cell cycle distribution after 24 h of chronic anoxia and hypoxia compared to normoxia is shown.

In addition, the cell cycle distribution for cells reoxygenated after chronic anoxia and hypoxia was measured over a 24-h period and compared to normoxic cells. The results show a block in G1, a smaller block in G2 and a strong reduction of S-phase cells directly after chronic anoxia. The distribution changes rapidly in the first 12 h and is comparable to aerobic cells after 15–20 h. In contrast, for

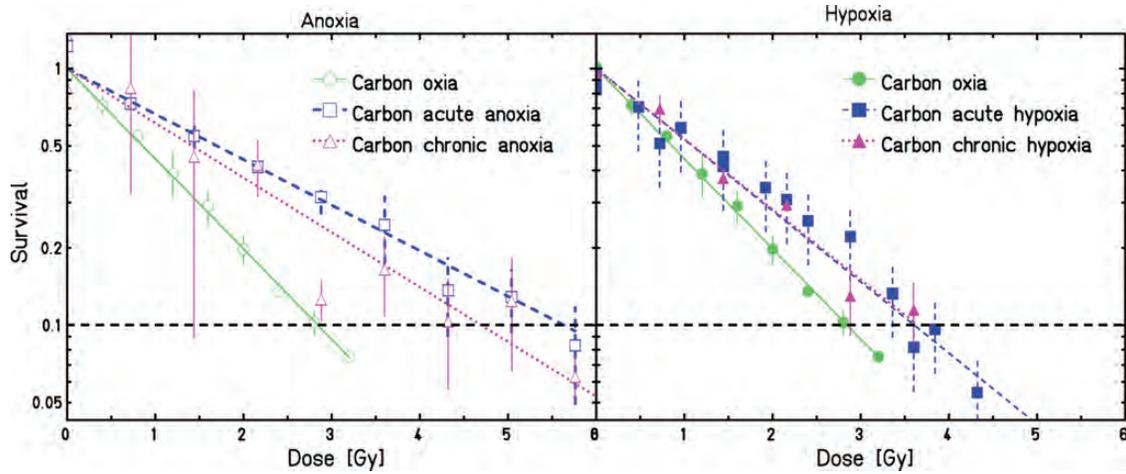


Fig. 2. Survival of CHO-K1 cells after carbon ion irradiation under different states of oxygenation. Data for acute states are from (W. Tinganelli *et al.*, submitted for publication) (simultaneous experiments).

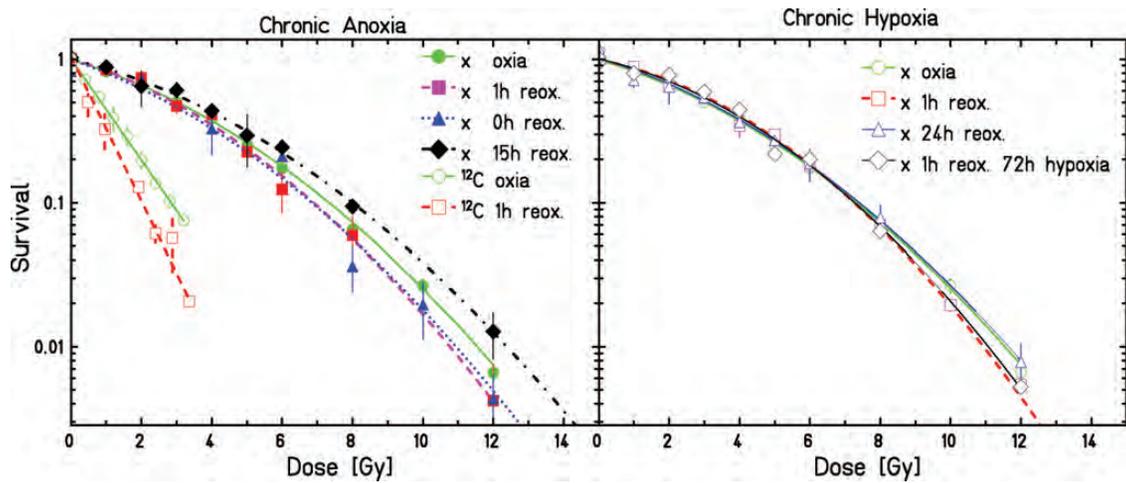


Fig. 3. Survival of CHO-K1 cells after irradiation under normoxia and at different times of reoxygenation from chronic anoxia or hypoxia. Data for acute states are from (W. Tinganelli *et al.*, submitted for publication).

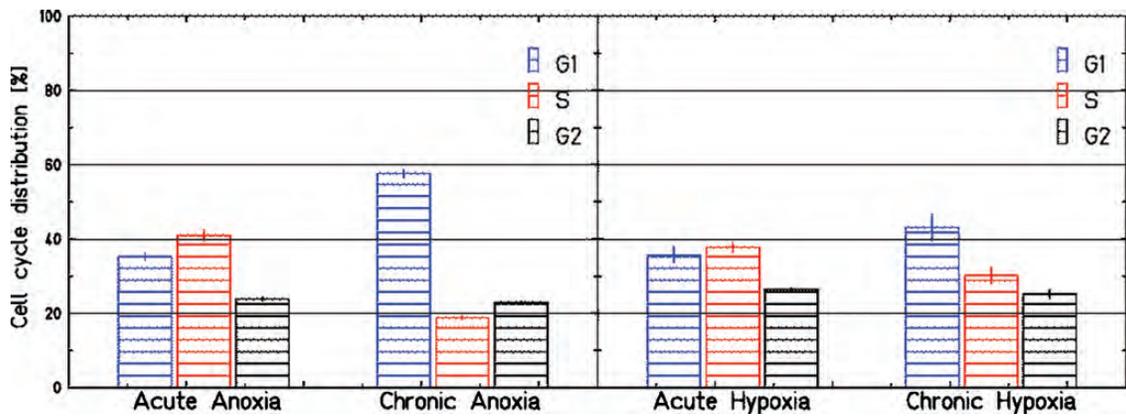


Fig. 4. Cell cycle distribution in CHO-K1 cells under acute and chronic anoxic and hypoxic conditions.

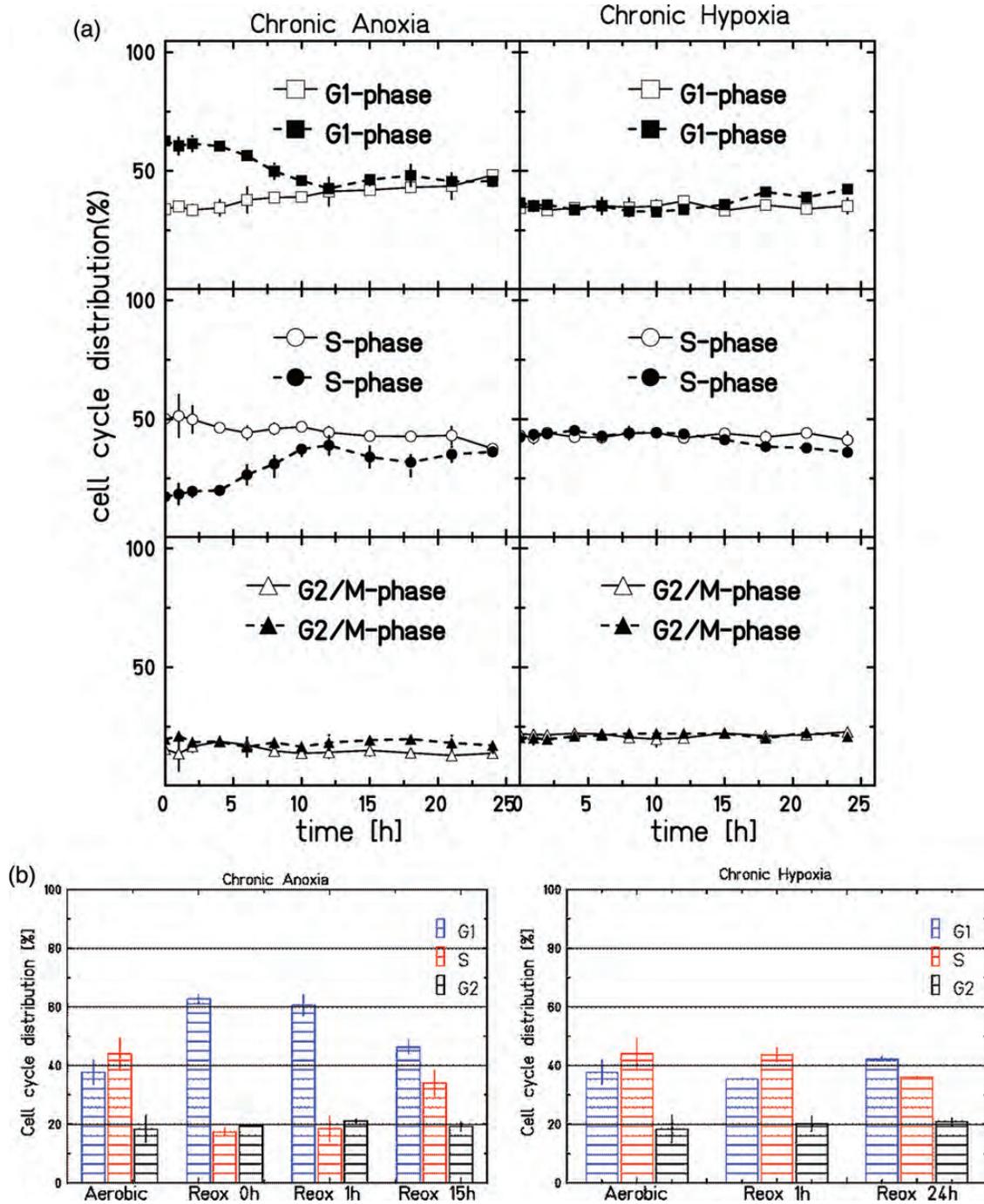


Fig. 5. Cell cycle distribution in CHO-K1 cells (A) during reoxygenation after chronic anoxia and hypoxia (closed symbols) compared to normoxic conditions (open symbols). (B) under reoxygenation after chronic anoxia and hypoxia. Time-points correspond to the survival curves in Fig. 3.

cells reoxygenated after chronic hypoxia a fairly uniform distribution was measured, which was similar to control cells (Fig. 5a, 5b).

To determine the influence of irradiation on the cell cycle, X-ray irradiation was performed with 2 and 6 Gy

under oxic conditions, and 2, 6 and 11.6 Gy under chronic anoxia. This reduced the cell survival to 64 and 18%, and 80, 50 and 18%, respectively. The induced cell cycle distribution was measured over 24 h (Fig. 6). After X-ray irradiation under aerobic conditions, a dose-dependent reduction

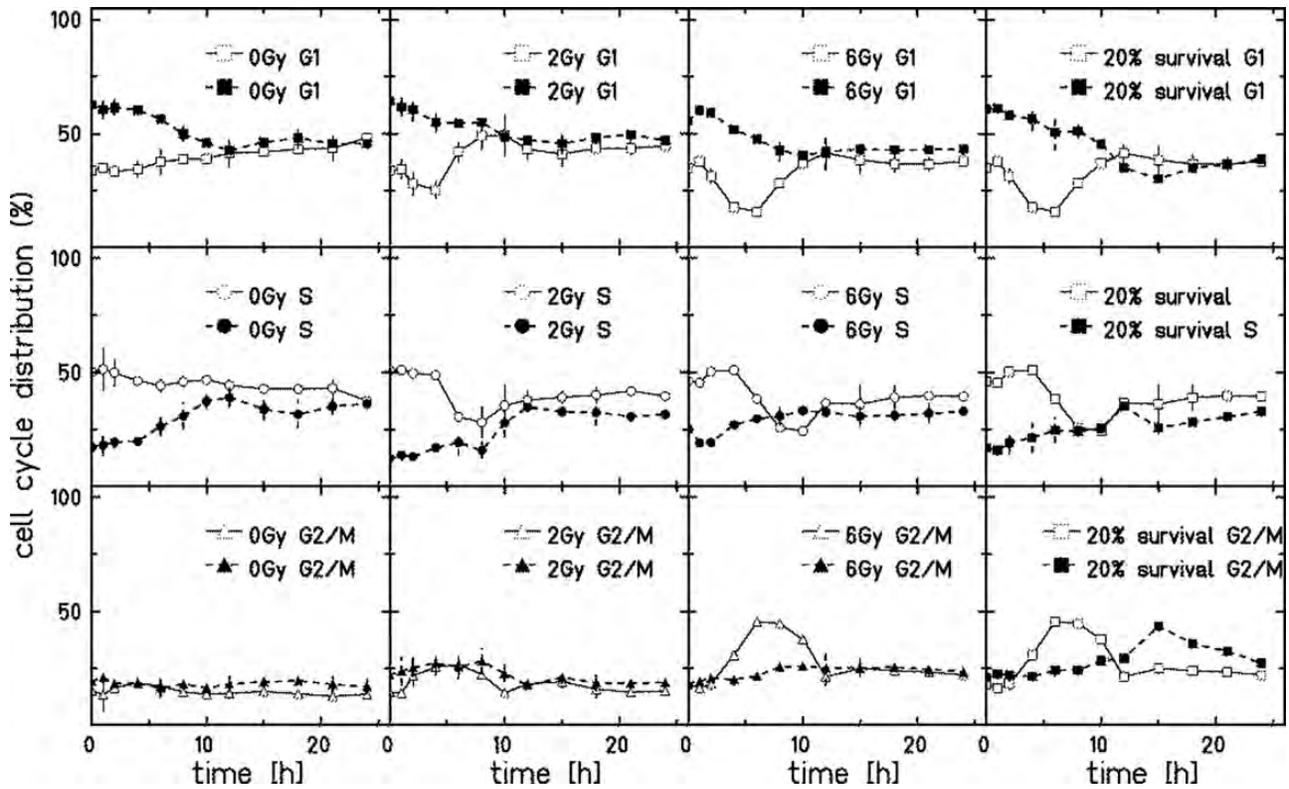


Fig. 6. Cell cycle distribution after X-ray exposure under normoxia (open symbols) or chronic anoxia (closed symbols). In the last column to the right, data are shown for cells exposed to 6 Gy (normoxic) or 11.6 Gy (chronic anoxia), corresponding to the same survival level.

of cells in G1 and S phase, and a dose-dependent accumulation of cells in G2/M phase resulting in a G2-block of several hours were found. The distribution of irradiated cells treated with 2 and 6 Gy after chronic anoxia changed rapidly in the first 12 h in a way similar to the unirradiated cells without a detectable dose-dependence, and was comparable to normoxic cells after 15–20 h. Irradiation with 11.6 Gy induced a small G2-block after 12–20 h.

DISCUSSION

We found that radiosensitivity depends on the time of oxygen deprivation: a small, but significant difference in sensitivity was found for chronic—compared to acute—anoxia. Cells irradiated under chronic anoxia are more sensitive compared to acute anoxia, indicating that the deprivation of oxygen is triggering two opposite effects: a ‘chemical effect’, which reduces the diffusion of the free radicals not fixed by the oxygen [19], and then leads to a higher survival; and a ‘biological effect’, producing changes in the cell metabolism [20], reducing the entity of the chemical effect. The latter can be seen in the smaller number of cells starting to proliferate again, which is expressed by the very low plating efficiency. This effect

reduces the X-ray OER_{10} from 2.3 under acute to 2.0 under chronic anoxia (Table 3). Chronic hypoxia, with 0.5% oxygen, did not show an equivalent effect in CHO-K1 cells. Real-time imaging showed that 24 h under 0.5% oxygen did not significantly affect cell proliferation. OER values were around 1.4 under acute as well as under chronic conditions. CHO-K1 cells seem to be more resistant to hypoxia, and sensitive only to values $<0.5\%$ oxygen, and thus behaved more like tumor stem cells than like normal cells. Additionally, previous chronic anoxic cells which were irradiated in the first 2 h of reoxygenation showed enhanced radiosensitivity compared to normoxia.

Irradiation with carbon ions with a LET_D of 100 keV/ μ m show similar results. Under anoxic conditions the OER is reduced from 2 under acute anoxia to 1.7 under chronic anoxia. The difference in OER between acute and chronic oxygen depletion seems to be in the same order and independent from the radiation quality. Again in the corresponding hypoxic case of 0.5% oxygen tension, the oxygen enhancement ratio decreases to $OER=1.3$, and does not differ for chronic or acute oxygen depletion. The sensitivity of reoxygenated cells is enhanced similarly for both C-ions and X-rays.

Possible reasons for the decreased radioresistance under chronic anoxia could be general metabolic changes and

changes in the cell cycle distribution, which strongly depends on the oxygenation status. It is evident that a sudden change in the oxygen tension—i.e. acute hypoxia or anoxia—cannot change the distribution of the cells in their cycle, which has a turnover time of many hours. Therefore, in the situation of acute changes, the cell cycle distribution stays close to the uniform distribution of normoxic cycling cells. A uniform distribution of cell cycle under hypoxia and reoxygenation after chronic hypoxia resulted in similar survival for both acute hypoxia and chronic hypoxia between 1 h and 24 h of reoxygenation.

In contrast, 24 h of anoxia induced an arrest in cell cycle progression, a block in G1 and a strong reduction of S-phase cells. An influence of the oxygen tension on the cell cycle has been reported by several authors. Spiro *et al.* [21] analyzed cell cycle distributions with bromodeoxyuridine (BrdU)-labeled V79 cells, finding a nearly complete arrest in G1 and S, but not in G2. Similar results were seen with the G1 phase and early and mid S phase of synchronized NHIK 3025 cervical carcinoma cells, which were completely arrested under hypoxic conditions [22]. A slightly different behavior has been described in xenografts from six different human tumors in nude mice [23]. The fraction of quiescent S-phase cells was correlated to the oxygenation levels. Inactive S-phase cells have also been reported in a study with p53 mutant human tumor cell lines where chronic hypoxia has been shown to delay G1 and S phase. The increased radiosensitivity over time under chronic hypoxia was regarded as a result of a breakdown in the cellular energy metabolism, which was attributed to the occurrence of inactive S-phase cells [7].

Freyer *et al.* [24] found a lower OER for G1 phase (2.3–2.4) compared to G2 phase (2.6–2.7) or cells in S phase (2.8–2.9). These data from literature confirm our measured imbalance in cell cycle distributions after chronic oxygen depletion. The reduced OER for chronic anoxia in our X-ray measurements could then be interpreted as the OER for a cell population that consists mainly of G1 cells, which are in general more sensitive than S cells.

At 0 and 1 h of reoxygenation after chronic anoxia the cell cycle distribution was the same as under chronic anoxia, and the high number of G1 cells was not reduced in this first stage of reoxygenation. Cell survival was decreased compared to constant normoxia, and the low plating efficiency showed that only a subpopulation survived 24 h of chronic anoxia. The reduced cell survival could be explained with the generally higher radiosensitivity of G1 cells to X-ray irradiation compared to S cells, and thus it might be more appropriate to compare these reoxygenated populations to chronic anoxia to calculate the OER. With this procedure, OER is 2.1 with only slight dependence on survival level. This is still smaller than the OER under acute anoxia and is more comparable to the results reported in reference [24].

Cell cycle progression starts again after ca. 1 h of reoxygenation, and after 15 h the cell cycle distribution is similar to oxic cells again. Cell survival measured 15 h after reoxygenation showed no decreased sensitivity compared to normoxic cells. Similar results were found in human squamous carcinoma cells irradiated after 10 min and 12 h of reoxygenation [25]. The slightly higher survival could then probably express a higher radioresistance of the surviving subpopulation compared to the original CHO-K1 line, as has also been found in glioma cells [26].

One rationale for introducing heavy ions into radiotherapy is the declining OER by increasing LET [15–18]. It has been previously shown that the yield of DNA double-strand breaks depends on oxygen concentration, but is the same after X-rays and C-ions [27], whereas rejoining kinetics reflects the OER [28]. We found a reduction of the OER after irradiation with carbon ions with a dose averaged LET_D of 100 keV/ μ m under acute anoxia (W. Tinganelli *et al.*, submitted for publication), but irradiation with the same beam under chronic anoxia and after 1 h of reoxygenation produced a similar decrease in radioresistance for X-ray irradiation. As the cell cycle dependence of cell inactivation is definitely reduced with increasing LET [15, 29], the difference in cell cycle distribution cannot be the only explanation for this increased sensitivity. Other reasons may be metabolic changes due to the reduced oxygen pressure. A range of these changes are reported in more recent literature, e.g. Kato *et al.* [30] found that the radiosensitivity of chronic hypoxic cells was significantly enhanced by reoxygenation; whereas in acute hypoxia the mRNA expression of BRCA1 and BRCA2 was reduced in cancer cells, reoxygenation increased the expression of BRCA1 and BRCA2. The hypoxia-inducible factors HIF-1 α and HIF-2 α , critical mediators of the hypoxia response, act differently in acute and chronic hypoxic conditions: HIF-1 α is more active in acute and transient hypoxia, whilst HIF-2 α is activated after 24 h of chronic hypoxia in most cell types [31]. The induced metabolic changes should therefore influence radiosensitivity to both high- and low-LET radiation in a similar way. In summary, these experiments showed that chronic anoxia reduced radioresistance compared to acute anoxia. For X-ray irradiation this can be explained partially by the strong reduction of S-phase cells or, more probably in compact tumors, by the high proportion of quiescent S-phase cells. The difference in OER was in the range of 10–20%. For adapted treatment planning this may play a minor role as the differences due to changes in the oxygen pressure in the different parts of the tumor will certainly be higher [32].

Reoxygenation induced a sensitivity, which for a time was also increased compared to normoxic cells. In radiotherapy, fractionated irradiation is normally one source of reoxygenation. As in an irradiated hypoxic tumor region the time of reoxygenation is greatly prolonged compared to

cell culture, there could be a window for enhanced radiosensitivity during one of the next fractions.

The enhanced sensitivity after reoxygenation was also seen for carbon irradiation. Therefore the benefit from fractionated irradiation could also be expected for particle irradiation.

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