The Therapeutic Effect of Photon Irradiation on Viable Glioblastoma Cells Is Reinforced by Hyperbaric Oxygen

HELMUT BÜHLER[1], GERFRIED L. STROHM[2], PASCALINE NGUEMGO-KOUAM[1], HANS LAMM[3], KHASHAYAR FAKHRIAN[2] and IRENÄUS A. ADAMIETZ[2]

[1]Institute for Molecular Oncology, Radio-Biology and Experimental Radiotherapy, and [2]Department of Radiotherapy and Radio-Oncology, University Medical Centre Marienhospital, Ruhr-University Bochum, Herne, Germany;
[3]Druckkammerzentrum Hannover, Hannover, Germany

Abstract. *Background: Hyperbaric oxygen (HBO) seems to intensify the effect of ionising radiation. We investigated whether HBO combined with irradiation decreases the capability of U251 glioblastoma cells for relapse and metastasis. Materials and Methods: Cells were treated with O_2 at 1.3 bar and then irradiated with 2 Gy photons. Clonogenic survival was tested with colony formation. Motility is an important feature of metastasis and was measured with time-lapse videography. Results: The clonogenic survival diminished by 22% through HBO, by 49% through irradiation, and by 70% through the combination of both. The accumulated distance travelled by cells fell by 3% with HBO, rose by 17% with irradiation, but was reduced by 11% with their combination. The respective values for the Euclidean distance travelled were +8%, +47% and −14%. Compared to normoxic irradiation, additional HBO lowered travel by 41%. Conclusion: HBO strengthens the effect of irradiation on clonogenic survival and reverses radiation-induced increase in the mobility of cells.*

Glioblastoma multiforme (GBM) is a serious malignant disease of the brain with a very poor survival prognosis. The therapeutic measures available, such as surgery, radiotherapy and systemic therapy, have only been able to improve relapse and survival rates to a small extent (1). There is evidence that the efficacy of ionising radiation can be increased through the use of hyperbaric oxygen (HBO) (2, 3). It is assumed that this effect of oxygen is caused by reinforced formation of reactive oxygen species (ROS) and free radicals in the cell (4). Moreover, solid tumours are characterised by the hypoxic conditions within them, conditions which suppress apoptosis, promote neoangiogenesis and generally induce a more aggressive phenotype of tumour cells, with increased invasiveness and metastasis (5-7). Moreover, hypoxia increases the resistance of tumour cells to irradiation (8). HBO can help to provide these hypoxic areas better with oxygen and hence improve the success of radiotherapy. In clinical application, HBO is largely safe and free of side-effects (9, 10). Earlier fears that increased partial pressure of oxygen may also bring about increased proliferation of tumour cells have not been confirmed (11, 10).

In vitro studies show that HBO not only has an antihypoxic effect, but also induces apoptosis of normoxically cultivated tumour cells and can hinder their proliferation (12). For GBM, there are only few data, and the data are partly contradictory (13, 14).

For this reason, we investigated two characteristics which, taken together, characterise the aggressiveness of tumour cells. By means of a clonogenicity test (colony formation), the relapse potential of the cells was estimated. The motility of tumour cells is related to their metastatic potential, and was determined through time-lapse videography of living cells. Both characteristics were investigated under conditions of both normal and increased partial pressure of oxygen.

Materials and Methods

Cell culture. The experiments were carried out with the established glioblastoma cell line U251 MG (ATCC, Manassas, VA, USA), cultivated in an incubator with 5% CO_2 in air at 37˚C. The culture medium was Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and penicillin/streptomycin (Biochrom, Berlin, Germany). The cells were cultivated in 25 cm^2 flasks (Sarstedt, Nümbrecht, Germany) and were passaged once a week. The culture medium was changed each time between the passages.

Correspondence to: Dr. Helmut Bühler, Universitätsklinikum Marienhospital, Hölkeskampring 40, D-44625 Herne, Germany, Tel: +49 23234991058, Fax: +49 23234991059, e-mail: helmut.buehler@rub.de

Key Words: Glioblastoma, hyperbaric oxygen, HBO, clonogenicity, motility, time-lapse videography.

Incubation with HBO. Culture flasks with cells of approximately 70% confluence were introduced into the pressure chamber, a custom-made item from Haux-Life-Support (Karlsbad, Germany). The gas-tight cap of the flasks was loosened prior to this, so that free gas exchange was possible. Pure O_2 was passed through the apparatus for 30 minutes in order to eliminate the normal ambient air. The oxygen pressure was then raised to 1.3 bar and the cells were incubated for 30 minutes. After careful decompression, the bottles were closed tightly so that the O_2 atmosphere was maintained. If irradiation of the cells was planned, this was carried out a few minutes after the end of the incubation with oxygen. An identical procedure was carried out with the control cells, except that they were not incubated with O_2 or were not irradiated.

Irradiation. Cells of approximately 70% confluence were irradiated in their culture flask with 2 Gy photons. A therapeutic linear accelerator Synergy S was used (Elekta, Hamburg, Germany), at a dose rate of 5 MeV/min. After the irradiation, the cells remained in the incubator for 3 hours before they were trypsinised, counted, and plated for further studies.

Clonogenicity test. In order to test their colony-forming ability, the cells were placed in six-well plates, at a density of 150 cells per well. After eight days, the medium was removed and cells were washed with phosphate buffered saline. The cells were fixed and stained with a solution of 2% crystal violet in methanol. After washing and drying, the number of colonies numbering more than 50 cells was counted with a microscope.

Videography. The motility of the cells was observed and documented using 24-hour time-lapse videography. The videography system is our own construction, based on a Zeiss Axiovert 25 (15). A holder is moved three-dimensionally by three precise linear motors and picks up either multiwell-plates or, with the help of a bracket, 3 cm Petri dishes, and allows their temperature to be maintained at 37˚C, in a constant atmosphere of 5% CO_2 in air. Any number of positions can then be defined at which the system, at freely selectable time intervals, travels to and takes a photograph.

The cells were disseminated at a density of 3×10^4 cells in Petri dishes and were kept for 6 hours in the incubator until all cells had become adherent. After this, they were transferred into the videography system, and in each of six dishes, two fields of view were selected for documentation, each with approximately 30 cells. Over a period of 24 hours, each field of view selected was photographed consecutively every 15 minutes.

Data analysis. In the sequences of images, the path of the cells was tracked with ImageJ (http://imagej.nih.gov/ij/) and was then evaluated with the Chemotaxis and Migration Tool provided by ibidi (http://ibidi.com/software/chemotaxis_and_migration_tool/). The following characteristic parameters of motility were analysed: total accumulated distance, Euclidean distance (geometrical displacement between the start and end points), and directness (Euclidean distance divided by total accumulated distance). A total of 20 cells per field of view were respectively analysed, having been selected in advance using the first image of each series.

The statistical evaluation was carried out with GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). The calculation of the significances was carried out with the unpaired *t*-test, as a two-tailed *p*-value.

Results

The colony-forming ability of the cells after the different forms of pretreatment is displayed in Figure 1. Compared with untreated cells, irradiation with photons reduced the number of colonies by 49% ($p<0.0001$). After 30 minutes of HBO, the number of colonies significantly fell by 22% ($p=0.0059$). When the cells were additionally irradiated with 2 Gy after HBO treatment, there was a reduction in the number of colonies of 70% compared with the untreated control ($p<0.0001$). Compared with the cells which were irradiated in ambient air, the HBO pretreatment brought about an additional significant 41% reduction in the number of colonies ($p=0.0004$).

The effect of HBO and irradiation on the motility of the cells was quantified using the parameters total accumulated distance and Euclidean distance with videography. Figure 2 shows an exemplary image from a sequence of 98 consecutive images of untreated cells. A total of 20 cells were tracked in each such image sequence and were analysed with the Chemotaxis Tool. Figure 3 shows the movement patterns of the cells having received different treatments. The tool places the starting points of the tracked cells together at one common origin. Here, the migration of 20 cells is displayed as an example. A total of 120 cells were analysed.

Untreated cells travelled a total distance of 655±27.4 μm within 24 hours (Figure 4A). After irradiation with 2 Gy photons, the cells were more active, and the total distance travelled rose by 17% ($p=0.0025$). Incubation with HBO for 30 minutes did not particularly change cellular migration. The total distance travelled fell by just under 3% ($p=0.6218$, n.s.). When irradiation was additionally carried out after the HBO treatment, the total distance travelled compared to the controls decreased by 11% ($p=0.0318$). Pretreatment with HBO is therefore able to fully prevent radiation-induced rise in motility. Compared with cells irradiated in ambient air, the HBO treatment reduced the distance travelled by 24% ($p<0.0001$).

The Euclidean distance, in other words the straight-line distance between the start and endpoint of cell migration, exhibited similar changes (Figure 4B). Irradiation increased compared with that for the control by 47% ($p<0.0001$). After pretreatment with HBO alone, the value rose slightly, by 8% ($p=0.4443$, n.s.). The additional irradiation after incubation with HBO brought about a 14% reduction of the Euclidean distance ($p=0.1133$, n.s.) compared with the untreated control. Compared with the value after irradiation in ambient air, incubation with HBO before irradiation brought about a reduction of 41% ($p<0.0001$) of the Euclidean distance travelled. For this motility parameter, incubation with HBO prior to irradiation entirely also prevents the radiation-induced rise which is observed in ambient air.

Taking the quotient of the Euclidean distance and the accumulated distance provides a measurement of the

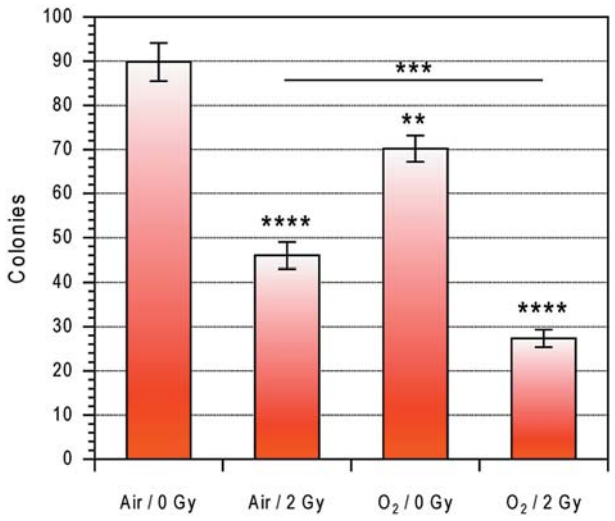

Figure 1. *Clonogenic survival in the colony-formation assay after hyperbaric oxygen (HBO) and irradiation. Human U251 glioblastoma cells were incubated for 30 minutes in 100% O_2 at an pressure of 1.3 bar and immediately afterwards were irradiated with 2 Gy photons. Untreated controls, as well as cells which were either only irradiated or only treated with HBO, were studied in parallel. The cells were plated at a density of 150 cells per well in 6-well plates and were cultivated for 8 days in an incubator. Afterwards the cells were fixed with MeOH and stained with crystal violet, and the number of colonies greater than 50 cells was determined. It became apparent that HBO treatment further reinforces the effects of irradiation and further reduces the number of colonies. HBO alone brings about a small, but significant reduction in the number of colonies. Each value displayed is the mean±S.E.M. Significant differences were calculated with the unpaired t-test of GraphPad Prism; n=6. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$, compared with the untreated control unless otherwise indicated.*

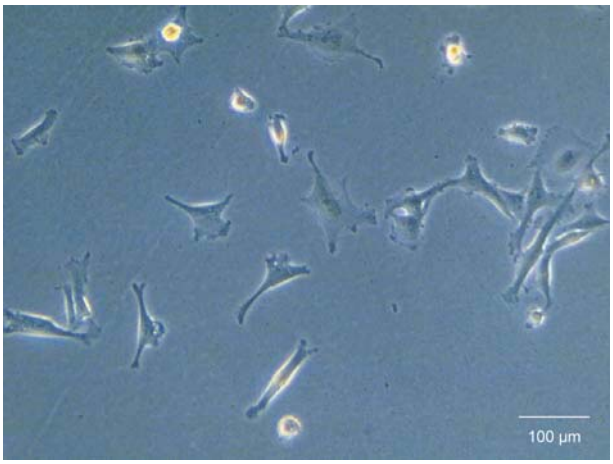

Figure 2. *A representative image from the videography sequence of untreated U251 cells. Dividing cells appear globular and highlighted under phase-contrast microscopy.*

directness of migration. The higher this value is, the fewer detours or erratic back-and-forth movements the cells make, and so the more target-oriented is their movement from their start to end point. The directness rose under normoxic irradiation (p=0.0050) (Figure 4C). After incubation with HBO, it remained slightly higher than that of the control (p=0.1150, n.s.) but was slightly lower than by normoxic irradiation. When treatment with HBO was additionally carried out before irradiation, directness did not differ from that of the control (p=0.7570, n.s.). Prior incubation with HBO entirely abrogated radiation-induced increase in directness (p=0.0313).

Discussion

Our experiments show that HBO restricts both clonogenic survival and the motility of cultivated GBM cells. The data were gained with vital cells, and therefore are more closely related to potential clinical applications than pure molecular biological or biochemical studies. There is a long history of the use of pure oxygen, both normal and hyperbaric, in cancer therapy (16). The results are contradictory, but successes in therapy have certainly also been reported (4, 9-11, 13, 17-20). Our studies were able to contribute towards an understanding of the underlying mechanisms.

Our data show that HBO on its own reduced the clonogenic growth of the GBM cells slightly. The effect was much stronger in combination with 2 Gy photons. Here we also found a significant advantage of this combination compared with irradiation alone in ambient air. Only few findings have been published on clonogenic growth. Most studies are concerned with proliferation or apoptosis. In most cases, clonogenic survival is impeded by HBO (21-24; reviewed in 25). The effects described tend to be smaller than in our studies. This may be due to the fact that we maintained the oxygen atmosphere during the irradiation through a gas-tight closure of the culture flasks. If cells are cultivated in Petri dishes, all of the oxygen has completely escaped by the time that irradiation begins. Here, the *in vitro* setting differs from the conditions in patients treated with HBO. In the organism, a higher partial pressure of oxygen can be demonstrated as long as 30 minutes after the end of the treatment (26, 27).

Our studies of cellular motility also showed an inhibitive effect of HBO, again, particularly in combination with irradiation. This is all the more important since irradiation with 2 Gy alone was found to increase the motility of GBM cells and therefore would increase the risk of metastasis. This effect, which we observed previously (28), has also been described by other groups recently (29-31).

We described the migration behaviour based on the two motility parameters of total accumulated distance and

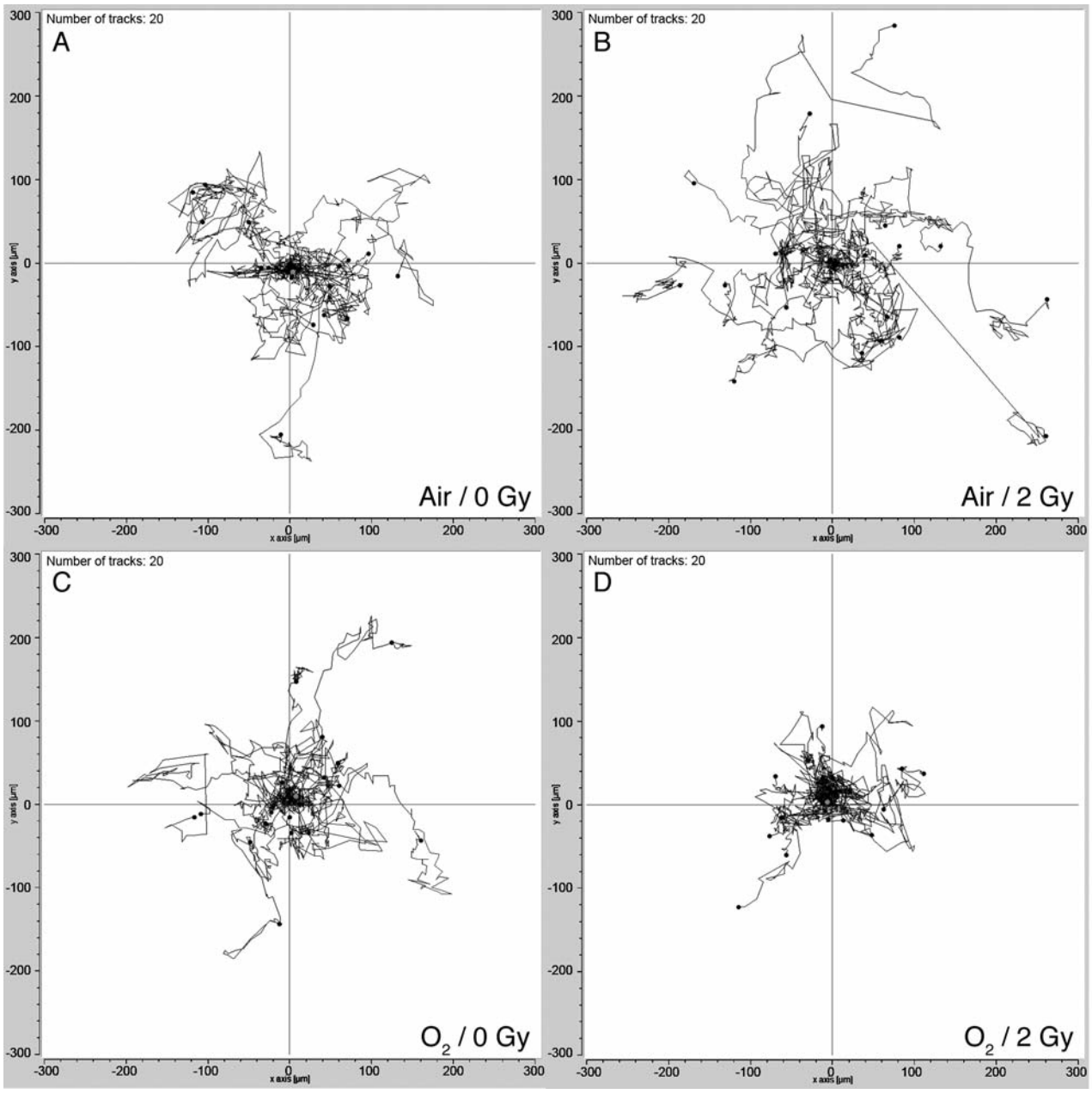

Figure 3. *Motility after hyperbaric oxygen (HBO) and irradiation. Human U251 glioblastoma cells were incubated for 30 minutes in 100% O_2 at an pressure of 1.3 bar, and immediately afterwards were irradiated with 2 Gy photons. Untreated controls, as well as cells which were either only irradiated or only treated with HBO, were investigated in parallel. A total of 30,000 cells were disseminated in 3 cm Petri dishes, and their movements were documented for 24 hours using time-lapse videography. Twenty selected cells were tracked in each image sequence and were analysed with the Ibidi Chemotaxis Tool. The figure shows as a representative graphic presentation of 20 tracked cells for each kind of pretreatment. The software places the starting point of all the cells at the origin of the graph so that whether the migration is directed and how strong the scatter of the cell migration is can be seen. Migration patterns are shown from untreated cells (A), cells which were irradiated in ambient air (B), cells under HBO without irradiation (C) and cells treated with irradiation with HBO (D). Even looking at the raw data, it is noticeable that without irradiation, there is scarcely any difference between the results in ambient air and in an oxygen atmosphere. However, clear differences become visible after irradiation with 2 Gy photons.*

Euclidean distance. This showed that HBO alone does not have any significant effect on the motility. Irradiation with 2 Gy alone increased both parameters. It was possible to entirely prevent this increase through prior treatment with HBO, and the values could even be reduced to values lower than those of untreated cells. In the literature, only few data can be found

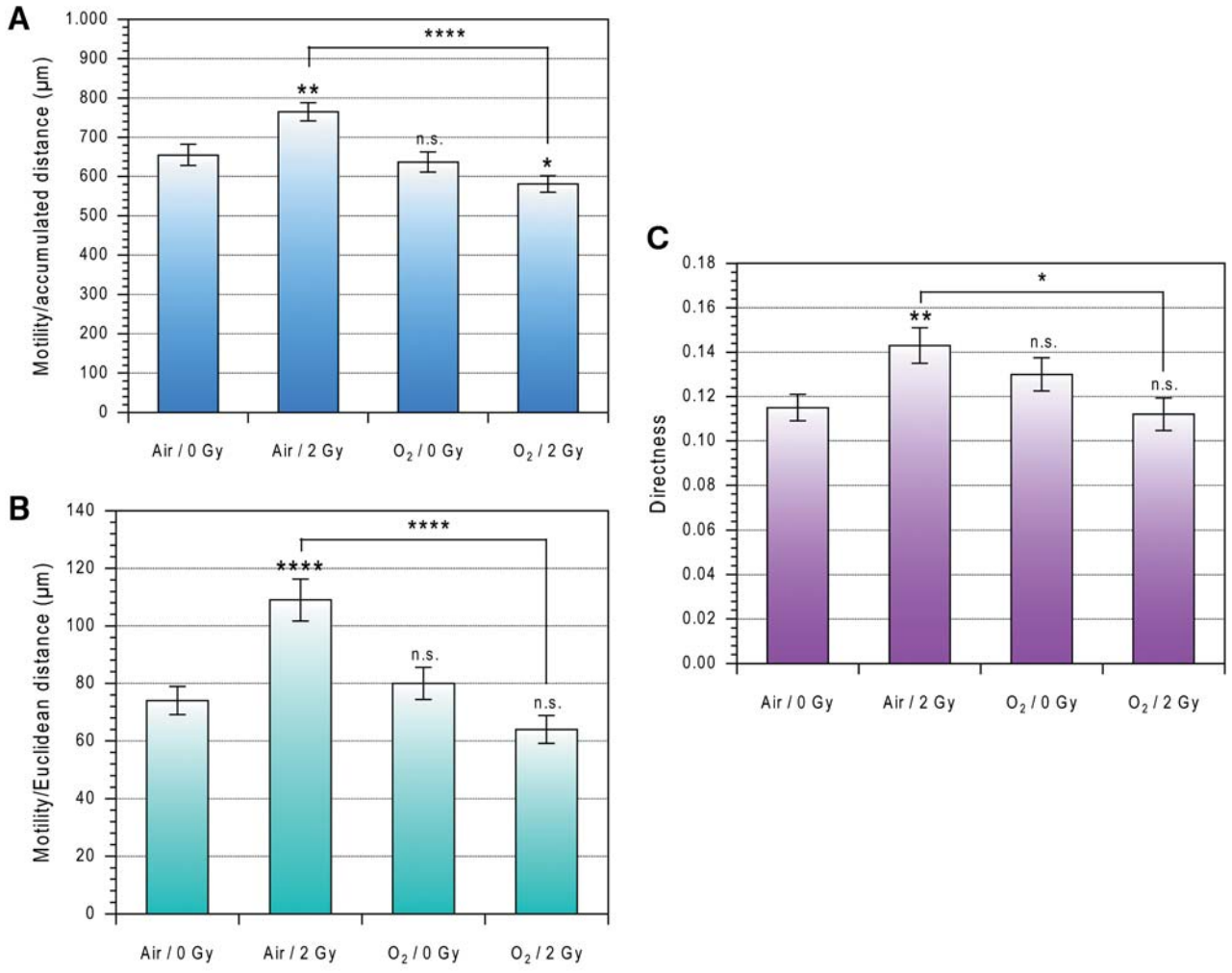

Figure 4. *Motility after hyperbaric oxygen (HBO) and irradiation. The experimental setting and analysis were as described in the legend to Figure 3. A: Evaluation of the total accumulated distance which the cells travelled on average over 24 hours. Irradiation with 2 Gy photons induces an increased migration of cells. Under HBO, migration is reduced and the radiation-induced rise is entirely prevented. B: Evaluation of the Euclidean distance travelled. The Euclidean distance is defined as the direct distance between the end point of the cell migration and its starting point, hence the real gain in distance of the cell. The progression is similar to that shown in part A, although the Euclidean distance after normoxic irradiation can be seen to rise even more strongly than the total accumulated distance. This rise is also fully blocked by HBO. C: The measure of directness, which is the quotient of the Euclidean distance and the accumulated distance, shows that following irradiation with 2 Gy photons, the cells travel in a straighter line and that the erratic back-and-forth behaviour is reduced. However, HBO treatment before irradiation entirely prevents this potentially metastatic effect. The graph displays the mean value±S.E.M. The significant differences were calculated with the unpaired t-test of GraphPad Prism on the basis of a total of 120 evaluated cells. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$, compared with the untreated control unless otherwise indicated. n.s.: Not significant.*

on the changing of motility under HBO. Peng et al. measured the migration of hepatoma cells in transwell experiments. No change was observed after HBO (12). Similar results were reported for nasopharyngeal cells: again no difference between controls and HBO-treated cells was observed (32). However, neither study really measured pure migration, but instead a combination of migration and chemotactic effects. An older study describes severely reduced motility after HBO treatment, however, not on malignant but sperm cells (33).

On the other hand, there are indications that HBO can increase cell migration. However, again these data are not for malignant cells but keratinocytes which are involved in wound healing (34).

We investigated the effect of HBO combined with photon irradiation with respect to two important properties of cancer cells which together provide information about the risk of a relapse: clonogenic survival as a predictive factor for local relapse, and migratory potential as a factor for distant

metastasis. By both, we found that the combination therapy of irradiation and HBO has an inhibitory effect, which may be the cause of the survival improvement through combined HBO and irradiation therapy which is described in the literature.

Acknowledgements

The Authors would like to thank Anja Kochanneck and Bettina Priesch for excellent technical assistance. They also gratefully thank the STIFTUNGSFONDS BIOX for supporting this work.

References

1 Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T,Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E and Mirimanoff RO: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med *352*: 987-996, 2005.

2 Kohshi K, Beppu T, Tanaka K, Ogawa K, Inoue O, Kukita I and Clarke RE: Potential roles of hyperbaric oxygenation in the treatments of brain tumors. Undersea Hyperb Med 40: 351-362, 2013.

3 Overgaard J: Hypoxic modification of radiotherapy in squamous cell carcinoma of the head and neck–a systematic review and meta-analysis. Radiother Oncol *100*: 22-32, 2011.

4 Gore A, Muralidhar M, Espey MG, Degenhardt K and Mantell LL: Hyperoxia sensing: from molecular mechanisms to significance in disease. J Immunotoxicol *7*: 239-254, 2010.

5 Harris AL: Hypoxia–a key regulatory factor in tumour growth. Nat Rev Cancer *2*: 38-47, 2002.

6 Thiery JP: Epithelial–mesenchymal transitions in tumour progression. Nat Rev Cancer *2*: 442-454, 2002.

7 Edsjö A, Holmquist L and Påhlman S: Neuroblastoma as an experimental model for neuronal differentiation and hypoxia-induced tumor cell dedifferentiation. Semin Cancer Biol *17*: 248-256, 2007.

8 Shannon AM, Bouchier-Hayes DJ, Condron CM and Toomey D: Tumour hypoxia, chemotherapeutic resistance and hypoxia-related therapies. Cancer Treat Rev *29*: 297-307, 2003.

9 Daruwalla J and Christophi C: Hyperbaric oxygen therapy for malignancy: a review. World J Surg *30*: 2112-2131, 2006.

10 Moen I and Stuhr LE: Hyperbaric oxygen therapy and cancer: a review. Target Oncol *7*: 233-242, 2012.

11 Feldmeier J, Carl U, Hartmann K and Sminia P: Hyperbaric oxygen: Does it promote growth or recurrence of malignancy? Undersea Hyperb Med *30*: 1-18, 2003.

12 Peng HS, Liao M, Zhang M, Xie Y, Xu L, Zhang Y, Zheng, X F Steven, Wang H and Chen Y: Synergistic inhibitory effect of hyperbaric oxygen combined with sorafenib on hepatoma cells. PLoS One *9*: e100814, 2014.

13 Kohshi K, Kinoshita Y, Imada H, Kunugita N, Abe H, Terashima H, Tokui N and Uemura S: Effects of radiotherapy after hyperbaric oxygenation on malignant gliomas. Br J Cancer *80*: 236-241, 1999.

14 Ogawa K, Ishiuchi S, Inoue O, Yoshii Y, Saito A, Watanabe T, Iraha S, Toita T, Kakinohana Y, Ariga T, Kasuya G and Murayama S: Phase II trial of radiotherapy after hyperbaric oxygenation with multiagent chemotherapy (procarbazine, nimustine, and vincristine) for high-grade gliomas: long-term results. Int J Radiat Oncol Biol Phys *82*: 732-738, 2012.

15 Bühler H, Adamietz R, Abeln T, Schneider O, Schilcher B and Adamietz IA: Building a system for time videography migration of glioblastoma cells under radiotherapy. Strahlenther Onkol *185*: 162, 2009.

16 Gill AL and Bell CNA: Hyperbaric oxygen: its uses, mechanisms of action and outcomes. QJM 97: 385-395, 2004.

17 Heys SD, Smith IC, Ross, J A S, Gilbert FJ, Brooks J, Semple S, Miller ID, Hutcheon A, Sarkar T and Eremin O: A pilot study with long-term follow-up of hyperbaric oxygen pre-treatment in patients with locally advanced breast cancer undergoing neo-adjuvant chemotherapy. Undersea Hyperb Med *33*: 33-43, 2006.

18 Thom SR: Hyperbaric oxygen: its mechanisms and efficacy. Plast Reconstr Surg 127 *Suppl 1*: 131S-141S, 2011.

19 Al-Waili NS, Butler GJ, Beale J, Hamilton RW, Lee BY and Lucas P: Hyperbaric oxygen and malignancies: a potential role in radiotherapy, chemotherapy, tumor surgery and phototherapy. Med Sci Monit *11*: RA279-89, 2005.

20 Kohshi K, Yamamoto H, Nakahara A, Katoh T and Takagi M: Fractionated stereotactic radiotherapy using gamma unit after hyperbaric oxygenation on recurrent high-grade gliomas. J Neurooncol *82*: 297-303, 2007.

21 Hewitt HB and Sakamoto K: The comparative survival of clonogenic cells of a murine epithelioma after irradiation in mice breathing air, oxygen and carbon dioxide, or hyperbaric oxygen. Br J Radiol *44*: 457-463, 1971.

22 Overgaard J: Sensitization of hypoxic tumour cells–clinical experience. Int J Radiat Biol *56*: 801-811, 1989.

23 Hartmann KA, Carl UM, Plugge DC, Schneider CJ and Sminia P: Enhancement of radiosensitivity of rat rhabdomyosarcoma R1H with normobaric carbogen and hyperbaric oxygen (HBO) using conventionally fractionated irradiation. Br J Radiol *71*: 433-437, 1998.

24 Granowitz EV, Tonomura N, Benson RM, Katz DM, Band V, Makari-Judson GP and Osborne BA: Hyperbaric oxygen inhibits benign and malignant human mammary epithelial cell proliferation. Anticancer Res *25*: 3833-3842, 2005.

25 Busk M and Horsman MR: Relevance of hypoxia in radiation oncology: pathophysiology, tumor biology and implications for treatment. Q J Nucl Med Mol Imaging *57*: 219-234, 2013.

26 Kinoshita Y, Kohshi K, Kunugita N, Tosaki T and Yokota A: Preservation of tumour oxygen after hyperbaric oxygenation monitored by magnetic resonance imaging. Br J Cancer *82*: 88-92, 2000.

27 Beppu T, Kamada K, Yoshida Y, Arai H, Ogasawara K and Ogawa A: Change of oxygen pressure in glioblastoma tissue under various conditions. J Neurooncol *58*: 47-52, 2002.

28 Abeln T, Kochanneck A, Polz K, Demirel C, Bühler H and Adamietz IA: Enhanced migration of cultured glioblastoma cells following radio therapy. Strahlenther Onkol *187*: 123-124, 2011.

29 Stahler C, Roth J, Cordes N, Taucher-Scholz G and Mueller-Klieser W: Impact of carbon ion irradiation on epidermal growth factor receptor signaling and glioma cell migration in comparison to conventional photon irradiation. Int J Radiat Biol *89*: 454-461, 2013.

30 Simon F, Dittmar J, Brons S, Orschiedt L, Urbschat S, Weber K, Debus J, Combs SE and Rieken S: Integrin-based meningioma cell migration is promoted by photon but not by carbonion irradiation. Strahlenther Onkol, [Epub ahead of print], 2014.

31 Moncharmont C, Levy A, Guy J, Falk AT, Guilbert M, Trone J, Alphonse G, Gilormini M, Ardail D, Toillon R, Rodriguez-Lafrasse C and Magne N: Radiation-enhanced cell migration/invasion process: A review. Crit Rev Oncol Hematol *92*: 133-142, 2014.

32 Peng Z, Zhong W, Liu J and Xiao P: Effects of the combination of hyperbaric oxygen and 5-fluorouracil on proliferation and metastasis of human nasopharyngeal carcinoma CNE-2Z cells. Undersea Hyperb Med *37*: 141-150, 2010.

33 Mayevsky A, Bar-Sagie D and Bartoov B: Sperm cell motility as a new experimental model for toxicological studies. Arch Toxicol *Suppl 6*: 295-299, 1983.

34 Kairuz E, Upton Z, Dawson RA and Malda J: Hyperbaric oxygen stimulates epidermal reconstruction in human skin equivalents. Wound Repair Regen *15*: 266-274, 2007.

Received December 8, 2014
Revised January 15, 2015
Accepted January 19, 2015