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Distinct H_2O_2 concentration promotes proliferation of tumour cells after transient oxygen/glucose deprivation

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Abstract

A solid tumour undergoes ischemia/reperfusion due to deficient vascularization and subsequent formation of new blood vessels. This study investigated the effect of transient oxygen and glucose deprivation (OGD) on proliferation of C6 glioma cells. The cells were subjected to 18 h of OGD followed by reoxygenation in the presence of glucose and different extra-cellular H_2O_2 concentrations since H_2O_2 affects cell proliferation. After reoxygenation, the cellular H_2O_2 concentration was increased returning to control levels within 24 h. Within this period, increase in cell number and MTT-reduction were impaired. Regeneration was completed on the third day of reoxygenation. MTT-reduction increased faster than cell number, indicating an OGD-dependent up-regulation of protein expression. It is concluded that ischemia/reperfusion stress promotes proliferation of tumour cells. An essential factor is a distinct H_2O_2 concentration. Massive elevation as well as significant reduction of H_2O_2 concentration impairs the proliferation process.

Keywords: *Hydrogen peroxide, cell proliferation, tumour cells, oxygen/glucose deprivation*

Introduction

A rapid growing tumour undergoes a change of ischemia and reperfusion. Ischemic episodes are induced by deficient vascularization in solid tumours. Concomitantly, the oxygen partial pressure falls below 10 mm Hg [1]. Under this condition the transcription factor HIF (hypoxia-inducible factor) is activated [2]. This factor mediates the expression of several proteins. Among them the endothelial growth factor-A (VEGF-A) has been identified to promote the formation of new blood vessels. The new blood vessels migrate into the tumour mass and support reperfusion of the tumour tissue with oxygen and nutrients [3,4].

Most of the tumour cells survive the ischemic period by using anaerobic glycolysis for energy production. They take advantage from the HIF-dependent up-regulation of glucose transport and enzymes of the glycolytic pathway. Thereby, glucose is recruited from internal stores and macroautophagy [5,6]. With re-establishment of vascularization the tumour cells return to oxygen-consuming metabolism that supports tumour growth. Thus, change of ischemia and reperfusion is a normal process in the development of solid tumours [7]. However, the effect of transient ischemia on the proliferation rate of tumour cells is unclear.

Gliomas are the most common primary neoplasm in the brain [8]. More information about the development of gliomas is needed since conventional surgery, radio- and chemotherapy have failed to significantly improve the prognosis of patients with

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malignant astrocytomas [9]. To study the effect of transient ischemia on the proliferation of glioma cells, we exposed C6 glioma cells to 18 h OGD. Afterwards, cell proliferation and viability were evaluated over 3 days of cultivation in growth medium. It has been shown that tumour cell proliferation sensitively depends on H_2O_2 concentration [10]. Therefore, these experiments were performed at different H_2O_2 concentrations by applying glucose oxidase or TEMPOL.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), PBS, horse radish peroxidase (HRP), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma (Taufkirchen, Germany), penicillin/streptomycin solution and foetal bovine serum (FBS) were from Gibco BRL (Karlsruhe, Germany), glucose oxidase from Aspergillus niger was purchased from Fluka (Taufkirchen, Germany), Trypsin/EDTA from PAA (Cölbe, Germany), poly-d-lysine from Boehringer (Manncheim, Germany). Petri dishes were from Greiner (Frikenhausen, Germany) and TEMPOL from Calbiochem (Darmstadt, Germany). All other chemicals were of analytical grade.

Cell culture

C6 glioma cells were obtained from the European Collection of cell cultures (Porton Down, Wiltshire, UK) and used at passage 6. The cells were cultured in DMEM medium supplemented with 3% FBS and 1% (v/v) penicillin/streptomycin (growth medium) at 37°C in a humidified air atmosphere with 5% CO_2. Cell suspensions were prepared at the density of 5 * 10^3 cells/ml and were seeded in 2 ml medium per 35 mm Petri dish. Before the experiments, cells were cultured over 3 days in a CO_2 incubator in 5% CO_2 in air atmosphere at 37°C. During hypoxia the cells were incubated in a salt buffer solution containing: NaCl, 109.5 mM; KCl, 5.4 mM; $CaCl_2$, 1.8 mM; $MgSO_4$, 0.8 mM; HEPES, 25 mM; NaH_2PO_4, 0.91 mm. In this medium, cells were incubated in N_2 atmosphere (oxygen concentration less than 1% of air saturation as controlled by means of a Clark type electrode) at 37°C for 18 h. In parallel, as a control, cells were incubated in air atmosphere with 5% CO_2 in growth medium at 37°C. Afterwards, cells were reoxygenated in growth medium in air atmosphere with 5% CO_2 at 37°C. Additionally, glucose oxidase (GO) or Tempol were added to the growth medium to vary the concentration of reactive oxygen spices. In Figure 1 a scheme of the experimental set up is shown.

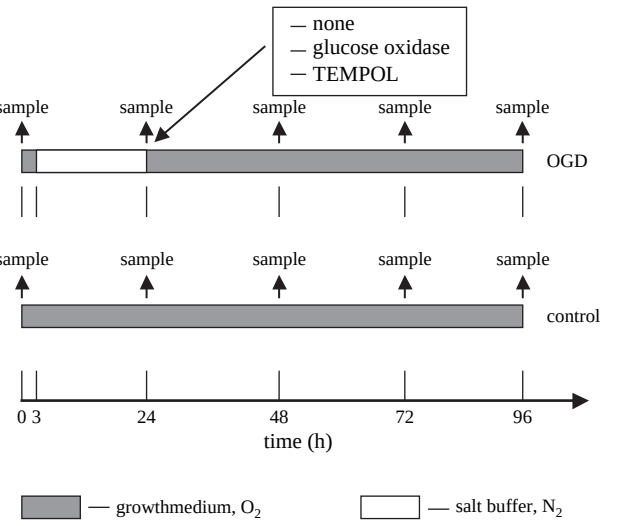

Figure 1. Scheme of the experimental set-up.

Quantification of cell proliferation

For the quantification of cell proliferation, cells were counted 6 h before and 1, 2 and 3 days after hypoxia. Hence the cells were washed twice with 2.5 ml PBS and treated with 500 µl trypsin for 3 min at 37°C. After addition of 500 µl PBS, 10 µl of the cell suspension were transferred into a Neubauer chamber and cells were counted. Two dishes of identical incubations were used for analysis. A total of five experimental series were used.

Determination of MTT reduction

Cells were incubated with 0.16 mg/ml MTT in a CO_2 incubator for 30 min at 37°C. MTT reduction was estimated by determining the quantity of formed formazan, which is the product of MTT reduction [11]. The change of absorption was determined at the wavelength of 570 nm in the presence of DMSO with 1 mM NaOH.

Determination of cellular H_2O_2

Cells incubated in Petri dishes were used after different times of cultivation for H_2O_2 imaging. Before imaging, the cells were incubated with DCFH-DA for 30 min after which they were washed once with HBSS buffer (HEPES buffer salt solution; NaCl 8.006 g/L, KCl 373 mg/L, $MgCl_2$ 0.182 mg/L, HEPES 4.76 g/L, glucose 991 mg/L, KH_2PO_4 81 mg/L, K_2HPO_4 0.01 mg/L, $NaHCO_3$ 840 mg/L and $CaCl_2$ 205 mg/L, adjusted to pH 7.4) to drain the excess dye. The cells were then visualized by means of the Zeiss Axiovert 100M Pascal confocal microscope. Each culture was subjected to five consecutive image acquisitions and the images were analysed for average fluorescent intensities of cells, taking background of each image as control. Each group consisted of five imaging experiments.

Determination of extra-cellular H_2O_2

For the determination of extra-cellular H_2O_2, 24 μM DCFH were added to Petri dishes after different times of cultivation. DCFH was obtained by alkalic splitting of DCFH-DA [12]. A specimen of 500 μl was withdrawn from the medium in the dish and supplemented with 3.75 mU horseradish peroxidise (POD). After 1 min vortexing, the sample was ready for fluorimetric analysis. Fluorescence intensity was analysed at 488 nm excitation-wavelength and 520 nm emission-wavelength.

Statistics

Data are presented as mean ± SEM of duplicates from five separate cell cultures. Statistical analysis was performed by using Student's paired *t*-test.

Results

Levels of H_2O_2 in C6 glioma cells after 18 h of OGD

To investigate the cellular level of H_2O_2 in C6 glioma cells after transient OGD, we exposed the cells to 18 h OGD followed by normoxic incubation in growth medium (reoxygenation). In Figure 1A, confocal microscope images of H_2O_2-dependent DCFH-fluorescence of control C6 glioma cells and C6 glioma cells treated with OGD are depicted. After 2 h of reoxygenation, treated cells displayed a significantly brighter DCFH-fluorescence in comparison to control cells (Figure 2A (reox 2 h vs control 2 h)). No difference in the images between controls and treated cells was found after 24 h of reoxygenation (Figure 1A (reox 24 h vs control 24 h)). The analysis of fluorescence intensities revealed the same tendency. The corresponding data are presented in Figure 2B. With reoxygenation, C6 glioma cells generated significantly more H_2O_2 in comparison to control cells. In particular, this was determined after 1 h as well as after 2 h of reoxygenation. In the further course of cultivation, the cellular H_2O_2 concentration returned to control levels. Thus, similar H_2O_2 concentrations were reached in control and in treated cells already after 24 h of reoxygenation. These data document the occurrence of oxidative stress in C6 glioma cells after OGD. However, these cells completely adapted within 24 h of reoxygenation.

Effect of transient OGD and H_2O_2 on the proliferation of C6 glioma cells

The extra-cellular H_2O_2 concentration was determined on the basis of H_2O_2-dependent DCFH-fluorescence. The sensitivity of this dye to H_2O_2 was checked by measuring the fluorescence intensity after addition of H_2O_2 solution to the incubation medium (Figure 3A). The stepwise elevation of the

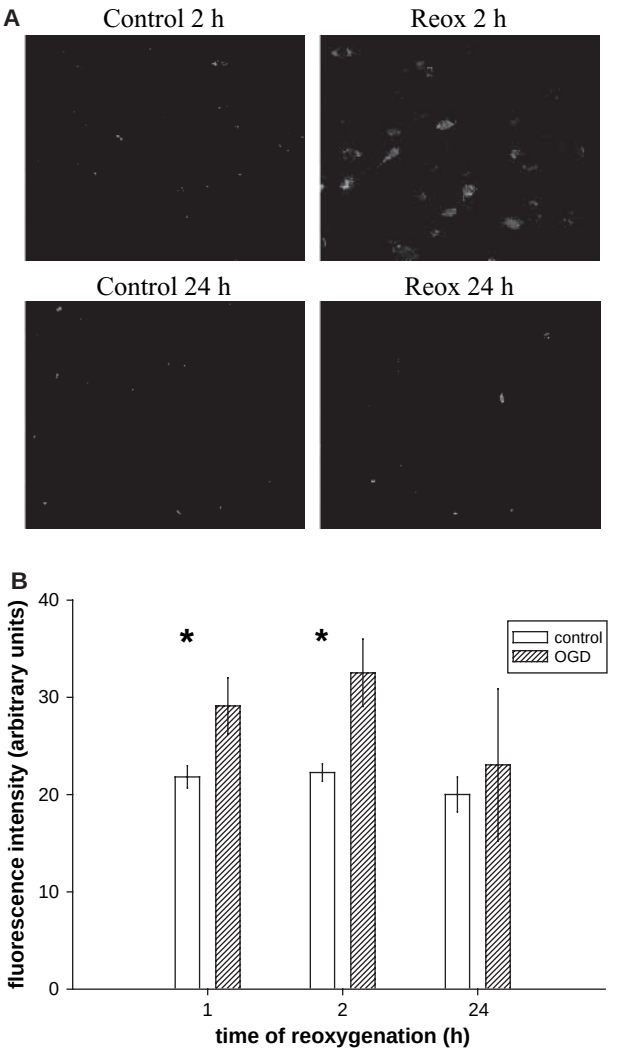

Figure 2. Effect of transient OGD on H_2O_2 generation in C6 glioma cells. C6 glioma cells were subjected to 18 h of OGD. After 1, 2 and 24 h of reoxygenation, samples were withdrawn for the analysis of intracellular H_2O_2 concentration by means of confocal microscopy. Before the analysis, cells were loaded with the H_2O_2-sensitive fluorescence dye DCFH. (A) Representative images of DCFH-fluorescence of control and treated cells are shown. Reox 2 h: cells after 2 h of reoxygenation, Control 2 h: control cells of the same age; Reox 24 h: cells after 24 h of reoxygenation, Control 24 h: control cells of the same age. (B) Fluorescence analysis. Average fluorescence intensities of cells taking background of each image as control were determined. The fluorescence data are based on five consecutive images from each of two cultures. *the difference was significant with $p<0.05$.

H_2O_2 concentration by 4.2 μM caused a similar increase in the fluorescence signal.

To study the influence of H_2O_2 on cell proliferation, we varied the concentration of H_2O_2 in the cultures of C6 glioma cells. Therefore, we applied two experimental approaches. First, the intracellular concentration of reactive oxygen species was reduced by applying the spin trap TEMPOL. This compound permeates the cell membrane and removes, dose dependently, superoxide anion radicals. Second, we added glucose oxidase of different activity to the culture medium in order to elevate the extra-cellular

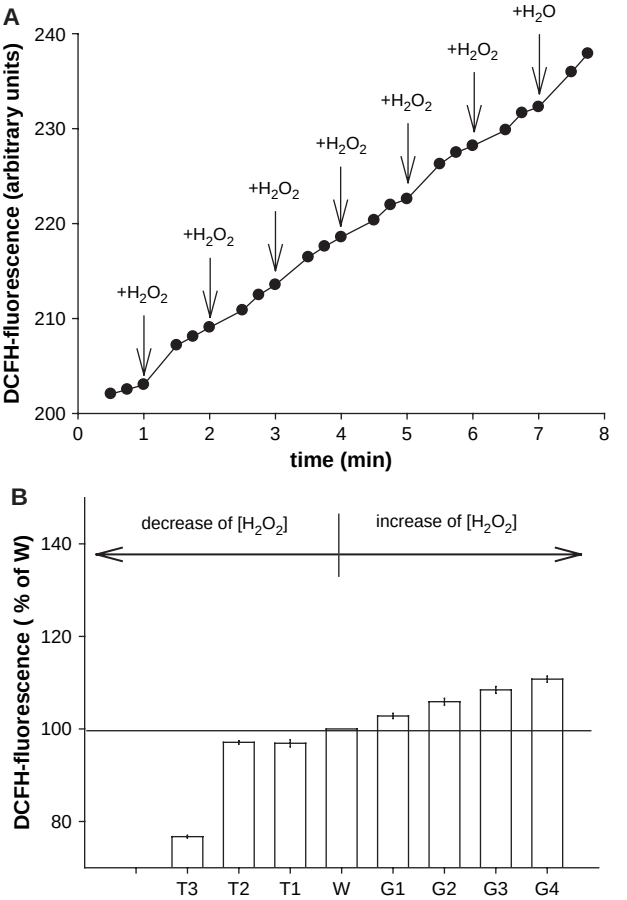

Figure 3. Adjustment of H_2O_2 concentration. Extra-cellular H_2O_2 was evaluated by H_2O_2-sensitive DCFH-fluorescence. (A) Sensitivity of DCFH-fluorescence to H_2O_2. At the indicated points (arrows) the concentration of H_2O_2 was elevated by 4.2 μM. Therefore H_2O_2 solution was added to the incubation medium. (B) Manipulation of the extra-cellular H_2O_2 concentration. 5000 glioma cells/ml were incubated in the presence of glucose oxidase plus glucose or TEMPOL. W: without additions; T1: 500 μM TEMPOL; T2: 1 mm TEMPOL; T3: 10 mm TEMPOL; G1: 0.5 mU/ml glucose oxidase; G2: 1.0 mU/ml glucose oxidase; G3: 1.5 mU/ml glucose oxidase; G4: 2.5 mU/ml glucose oxidase. Data are presented as mean ± SEM of six separate experiments.

H_2O_2 concentration. In Figure 3B the effect of this treatment on DCFH-fluorescence is presented. The horizontal line at 100% DCFH-fluorescence represents the concentration of H_2O_2 originated from the cells themselves (W). In the presence of glucose oxidase (G1–G4), an increase in the fluorescence signal was observed, indicating an increase in extracellular H_2O_2 concentration. On the contrary, TEMPOL (T1–T3) diminished, dose-dependently, the fluorescence signal, indicating a decrease in extracellular H_2O_2 concentration.

To study the effect of transient OGD and H_2O_2 on proliferation of C6 glioma cells, the cells were counted prior and after the treatment. In Figure 4A, the number of C6 glioma cells without (control) and with OGD-treatment (OGD) are presented. During OGD, cell proliferation was nearly stagnant due to the lack of oxygen supply and limited substrate availability. However, the cells survived the treatment. Within the first 24 h of reoxygenation, the proliferation rate was significantly lower in comparison to the control incubation. This period of time was characterized by elevated concentration of reactive oxygen species (see Figure 2B), possibly causing impairment of cellular function such as the proper operation of the cell cycle. Afterwards, the proliferation rate continuously increased, reaching nearly control levels at 72 h of reoxygenation.

To quantify proliferation of control cells and cells treated with transient OGD we took the cell number into account. Proliferation of cells was expressed by the change in cell number within 24 h. For comparison, control cell proliferation was estimated for identical start cell density by interpolation. Using this method, we studied the effect of transient OGD and H_2O_2 concentration on proliferation of C6 glioma cells. For better illustration, the changes in cell number of treated cells are related to corresponding changes in cell number of control cells and are expressed in percentage of control (Figure 4B). After OGD, the proliferation strongly depended on the extra-cellular H_2O_2 concentration. Low (T3) as well as high (G4, G5) extra-cellular H_2O_2 concentrations were accompanied by cell death as indicated by a decrease in cell number (negative changes in cell number). In a distinct range of H_2O_2 concentration (T1, W, G1–G3), proliferation was supported after OGD. In comparison to control cells, proliferation was remarkably reduced within the first 24 h of reoxygenation (24–48 h). However, within the following 2 days (48–72 h, 72–96 h) proliferation reached similar rates to control cells.

Similar results were obtained by analysing the ability of C6 glioma cells to reduce MTT to formazan after OGD. This parameter reflects not only the intactness of the plasma membrane and the number of cells but also the cellular redox power. In this context we tested the possibility that H_2O_2 can directly affect the MTT-assay. For this purpose, we separately exposed MTT and formazan to 100 μM H_2O_2 by using a H_2O_2 stock solution. We used C6 glioma cells in order to form formazan. To exclude interference with cellular metabolism, H_2O_2 was added after lyses of the cells. No effect of H_2O_2 on the absorption at 570 nm was observed. Thus, 100 μM H_2O_2 neither caused the reduction of MTT nor the degradation of formazan. MTT reduction of control and treated cells is presented in Figure 5A. For comparison with control incubations the same procedure of data analysis was applied as was used to create Figure 3B. The corresponding data are depicted in Figure 5B. In comparison to cell number, OGD caused in the presence of endogenous H_2O_2 smaller impairment of MTT reduction (50% ΔMTT reduction of control (W) vs 22% Δn of control (W))

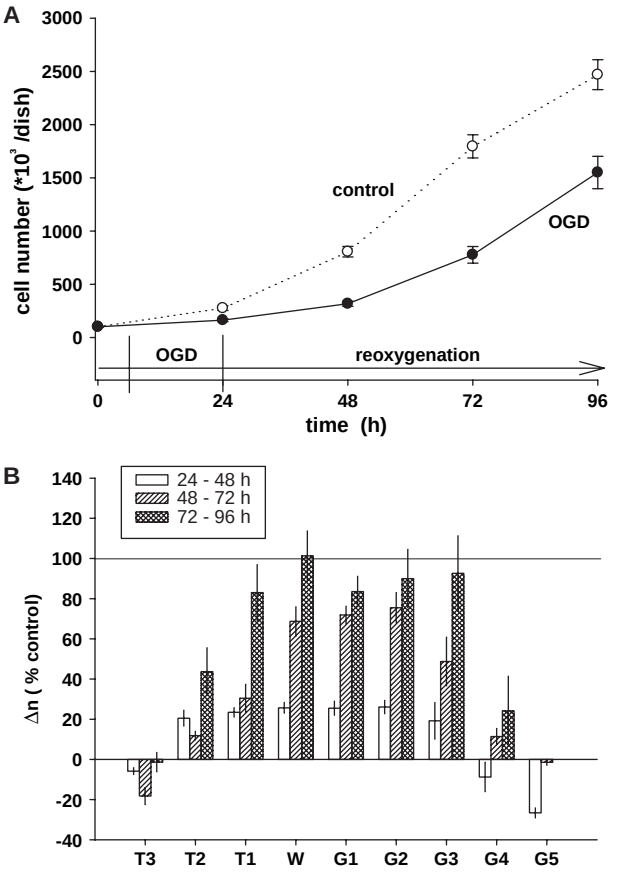

Figure 4. Effect of transient OGD and H_2O_2 on the proliferation of C6 glioma cells. After 3 days of cultivation cells were used for the experiment. The C6 glioma cells were subjected to 18 h of OGD. At the time of reoxygenation glucose oxidase or TEMPOL was added for manipulation of H_2O_2 concentration. Control cells were cultured under normal conditions. (A) Change in cell number in dependence on time of cultivation. Cell number was determined 6 h prior OGD (0 h), at the time of reoxygenation (24 h) and 24 h (48 h), 48 h (72 h) and 72 h after reoxygenation (96 h). (B) Relative change of cell number within 24 h (Δn). The change in cell number of treated cells was related to the corresponding change in the control incubation starting at the same cell number. Change in cell number was determined during the first, the second and the third day after reoxygenation. W: without additions; T1: 500 μM TEMPOL; T2: 1 mM TEMPOL; T3: 10 mM TEMPOL; G1: 0.25 mU/ml glucose oxidase; G2: 0.5 mU/ml glucose oxidase; G3: 1.0 mU/ml glucose oxidase; G4: 1.5 mU/ml glucose oxidase; G5: 2.5 mU/ml glucose oxidase. Data from six separate cultures are presented as mean ± SEM.

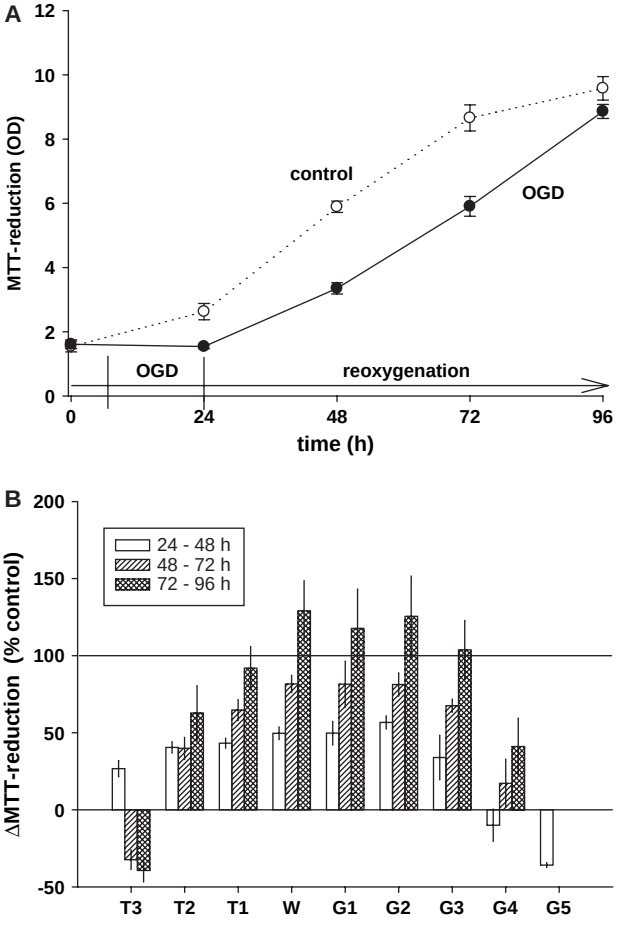

Figure 5. Effect of transient OGD and H_2O_2 on MTT-reduction of C6 glioma cells. After 3 days of cultivation cells were used for the experiment. The C6 glioma cells were subjected to 18 h of OGD. At the time of reoxygenation glucose oxidase or TEMPOL was added for manipulation of H_2O_2 concentration. Control cells were cultured under normal conditions. (A) MTT-reduction in dependence on time of cultivation. (B) Relative change of MTT-reduction within 24 h (ΔMTT). The change of MTT-reduction of treated cells was related to the corresponding change of control cells starting at the same MTT reduction. W: without additions; T1: 500 μM TEMPOL; T2: 1 mM TEMPOL; T3: 10 mM TEMPOL; G1: 0.25 mU/ml glucose oxidase; G2: 0.5 mU/ml glucose oxidase; G3: 1.0 mU/ml glucose oxidase; G4: 1.5 mU/ml glucose oxidase; G5: 2.5 mU/ml glucose oxidase. Data from six separate cultures are presented as mean ± SEM.

within the first 24 h of reoxygenation (24–48). In the presence of H_2O_2 concentrations supporting proliferation (W, G1, G2), the increase in MTT reduction was higher than under control conditions during the third day of reoxygenation (72–96), whereas increase in cell number only reached the level of control incubations. Thus, transient OGD increased the ability of C6 glioma cells to reduce MTT. These data point towards a metabolic adaptation process initiated by transient OGD.

Discussion

Oxidative stress has been demonstrated after reperfusion in many studies (for review see [13]). Besides elevated concentrations of reactive oxygen species (ROS), oxidative modification of lipids, proteins and DNA molecules have been reported [14–16]. In our experiments, elevated cellular H_2O_2 concentration within the first 24 h of reoxygenation was parallelled by significantly decreased proliferation rates. This may result from oxidative impairment of proteins, lipids and DNA molecules that are essential for cell proliferation. A further hallmark of non-lethal transient

ischemia is the up-regulation of genes which, in consequence, protect cells from ischemia/reperfusion injury (pre-conditioning). In this context, activation of the transcription factors HIF, NF-κB and AP-1, protein kinase C, Src protein tyrosine kinase and stimulation of the synthesis of inducible NO synthase, cyclooxygenase-2, aldose reductase and Mn superoxide dismutase have been demonstrated [17–20]. In our experiments, we have observed regeneration of cell proliferation within 3 days of reoxygenation. At the third day, we have detected considerable amounts of HIF at the RNA level. Since HIF is activated during hypoxia and deactivated in the presence of oxygen [21], the involvement of this signal molecule in the post-hypoxic regeneration is likely. In particular, HIF activation and possibly other signal cascades may mediate increased expression of proteins of the cell cycle that were impaired by oxidative stress.

A further essential result of our experiments is the different development of cell number and MTT-reduction. In comparison to control incubations, increase in MTT-reduction was less impaired than increase in cell number within the first 24 h of reoxygenation. Moreover, at the third day after reoxygenation, MTT-reduction increased faster than in the control incubation whereas increase of cell number only reached control levels. The ability of C6 glioma cells to reduce MTT was increased after OGD. We rule out a direct effect of H_2O_2 on MTT reduction under the experimental conditions since 100 μM H_2O_2 did not affect the MTT-assay. In the experiments, H_2O_2 concentration was always lower than 100 μm. Increase in MTT-reduction reflects a higher activity of cellular reductases that are able to reduce MTT to formazan. Based on this observation we speculate that transient OGD triggers an increase in the expression of cellular reductases and possibly other proteins that are involved in the regulation of cell proliferation. Our observations point towards an OGD-dependent change in the metabolism of C6 glioma cells. This change in the metabolism of C6 glioma cells after transient OGD may be essential for re-establishment of high proliferation rates and, possibly, for further stimulation of proliferation as an important aspect of accelerated tumour progression.

Our data further demonstrate that distinct H_2O_2 concentration is required for rapid proliferation after transient OGD. In solid tumours, characterized by high cell density, H_2O_2 generated by the tumour cells themselves is sufficient to promote cell proliferation. Our cell model mimics this situation. Both strong elevation of extra-cellular H_2O_2 concentration and significant reduction of cellular H_2O_2 concentration were accompanied by decreased proliferation rates. The sensitivity of the proliferation of tumour cells to H_2O_2 has been demonstrated for different cell types [22,23]. Here we demonstrate for the first time that H_2O_2 is involved in the process of regeneration of tumour cells after transient OGD. To discover the precise underlying mechanism of H_2O_2-dependent support of regeneration after transient ischemia of tumours, further investigations are required.

In vitro models such as cell cultures are helpful for the investigation of the influence of special factors on cell proliferation. Therefore, we used C6 glioma cells in order to study the effect of transient oxygen and glucose deprivation and H_2O_2 on proliferation of tumour cells. The supply with oxygen and substrates of tumour cells *in vivo* is dependent on the distance to the blood vessel. This heterogeneity is not covered by our cell model. We choose 18 h of oxygen and glucose deprivation since this treatment did not cause massive death of the glioma cells. The survival of transient OGD is typical for tumour progression [24]. On the other hand, the treatment resulted in significantly elevated cellular H_2O_2 concentration within the first 24 h after reoxygenation, which is known to occur in ischemia/reperfusion [25–27]. Two reasons led us to restrict the time of reoxygenation to 3 days. First, substrate supply was sufficient without change of medium. Second, the cell number did not significantly limit proliferation.

Based on our data we conclude that tumour cells are able to regenerate from ischemia/reperfusion stress within a few days. This type of stress initiates changes in the metabolism of tumour cells indicated by high degrees of MTT-reduction. An essential factor in the regeneration process is a distinct H_2O_2 concentration. Massive elevation of H_2O_2 concentration stops the regeneration process and tumour growth. Similar results can be achieved by reducing the H_2O_2 concentration, for example by application of sufficient amounts of antioxidants.

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