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Non-sufficient cell cycle control as possible clue for the resistance of human malignant glioma cells to clinically relevant treatment conditions

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Summary. *Objectives*. Human gliomas have a catastrophic prognosis with a median survival in the range of one year even after therapeutic treatment. Relatively high resistance towards apoptotic stimuli is the characteristic feature of malignant gliomas. Since cell cycle control has been shown to be the key mechanism controlling both apoptosis and proliferation, this study focuses on DNA damage analysis and protein expression patterns of essential cell cycle regulators P53 and $P21^{waf1/cip1}$ in glioma under clinically relevant therapeutic conditions.

Material and methods. U87MG cell line, characterised by wild *p53*-phenotype relevant for the majority of primary malignant glioblastomas, was used. Glioma cells underwent either irradiation or temozolomide treatment alone, or combined radio/chemo treatment. DNA damage was analysed by the "Comet Assay". Expression rates of target proteins were analysed using "Western-Blot" technique.

Results and conclusions. "Comet Assay" demonstrated extensive DNA damage caused by temozolomide treatment alone and in combination with irradiation, correlating well with the low survival rate observed under these treatment conditions. In contrast, irradiation alone resulted in a relatively low DNA damage, correlating well with a high survival rate and indicating a poor therapeutic efficiency of irradiation alone. Unusually low up-regulation of P53 and $P21^{waf1/cip1}$ expression patterns was produced by the hereby tested stressful conditions. A deficit in cell cycle control might be the clue to the high resistance of malignant glioma cells to established therapeutic approaches.

Keywords: Glioma therapy – Stress – DNA damage – Comet assay – Cell cycle control – Differential gene expression

Introduction

Human gliomas are the most frequent malignant brain tumours and the most malignant of the astrocytomas. A catastrophic prognosis of the median survival in the range of one year can be only marginally improved by current therapy approaches (Stupp et al., 2005; Wild-Bode et al., 2001). The excessive proliferation, disseminated tumour growth, extremely rich neovascularisation, and resistance towards apoptotic stimuli are the main features of malignant gliomas making their treatment especially complicate. The resistance of malignant glioma cells towards stress conditions results in sub-lethal effects under clinically relevant therapy approaches and, consequently, in an enhanced invasiveness of treated cells (Trog et al., 2006a, b). This scenario cannot lead to success in the therapy of malignant gliomas. In order to reconsider the current therapy approaches, some key mechanisms, which underline the response of glioma cells to treatment conditions, should be clarified. The clue is obviously a complex molecular and resulting cellular response to sub-lethal genotoxic conditions.

Animal experiments have shown a clear correlation between the expression level of drug resistance genes and sensitivity of oligodendrogliomas and astrocytomas to chemotherapy (Nutt et al., 2000). The inducible effect of both irradiation and chemo treatment on the expression level of ATP-binding cassette transporter 1 (ABC 1) in glioma cells has been recently demonstrated indicating the key role of drug resistance genes in the cross-resistance towards both irradiation and chemo treatment (Trog et al., 2005). The ABC 1 transporter connects functionally the cellular drug export and vascular blood–brain barrier regulation. There is a growing body of evidence that ABC 1 activation is induced by increased both DNA damage grade and expression levels of *p53*, which is considered to be one of the key regulators among drug resistance genes (Nutt et al., 2000). On its part P53 activity is regulated in response to genotoxic conditions such as irradiation and chemo treatment. Moreover, P53 expressional status has been recently proposed to be a predictor for therapy sensitivity in human malignant glioma cells (Hermisson et al., 2006).

Since the cell cycle control has been shown to be the key mechanism controlling both apoptotic and proliferation events under genotoxic conditions, we focused this study on DNA damage analysis and investigation of the corresponding regulation of protein expression patterns of cell cycle regulators P53 and $P21^{waf1/cip1}$ in glioma cells under clinically relevant therapeutic conditions. In this work we investigated the effect of irradiation and chemo treatment alone as well as the effect of combined chemo/radio treatment on human U87MG glioma cells. U87MG cell line is characterised by the wild *p53*-phenotype, which is relevant for the majority of primary malignant glioblastomas (Horiguchi et al., 2001). This cell line belongs to human astrocytes expressing vimentin relatively poorly with high basal expression levels of RhoA. From viewpoint of malignancy grade and consequently poor prognosis, the most unfavourable molecular interplay turns out, if vimentin is up-regulated, while RhoA is down-regulated; this is the case under the combined chemo/radio treatment of U87MG cell line (Trog et al., 2006a). This indicates the growing malignancy grade of the surviving cell fractions under combined chemo/radio treatment conditions, the phenomenon usually observed in later tumour reoccurrence after clinical gliomas treatment (Arab et al., 1999; Sallinen et al., 2000).

For the chemo treatment of U87MG cells temozolomide (TMZ) – a DNA-methylating agent – was used. TMZ produces O^6-methylguanine in DNA which mispairs with thymine in following cycles of DNA replication (Denny et al., 1994; Wedge et al., 1996). TMZ is currently used for treatment of recurrent high-grade gliomas and has been shown to yield objective response or stable disease in 50–60% of gliomas (Bower et al., 1997; Osoba et al., 2000; Paulsen et al., 1999; Yung et al., 1999). The function integrity of cell cycle checkpoints is assumed to be important in the cytotoxicity of TMZ in glioma cells (Hirose et al., 2001). We hypothesised here a functional overlap between cell cycle regulation and cross-resistance of the malignant gliomas towards both irradiation and chemo treatment introducing sub-lethal effects.

Materials and methods

Cell preparation and treatment

Human U87MG glioma cells were cultured at 37 °C in RPMI-1640 medium (Invitrogen, USA) supplemented with 10% fetal calf serum (Invitrogen, USA) and 1% Penicillin/Streptomycin (Invitrogen, USA). In parallel experiments the cells underwent either radiotherapy, (200 KV x-ray, MG 420 Philips) or chemo treatment with 30 µg/ml of TMZ in the cultivation medium, or combined chemo/radio treatment. The cell cultures were treated during 5 days from day 4 till day 9 of growth. Cell counting was performed using a Fuchs-Rosenthal counting cell chamber. After each treatment the surviving cell fractions were harvested, washed with PBS, aliquoted and stored at −80 °C until DNA and protein analysis. All experiments were repeated three times for the proper statistical evaluation.

"Comet assay" analysis

The single-stranded DNA damage was measured as increased migration of DNA using the single-cell gel-electrophoresis technique under alkaline conditions. The "Comet assay" (Trevigen Inc., USA) provides a simple and effective method for evaluation of DNA damage in cells. The principle of the assay is based upon the ability of DNA fragments to migrate out of the cell under the influence of an electric field. An evaluation of the "comet" tail shape and DNA fragments migration pattern allows for assessment of DNA damage.

In detail this method was described in literature (Lemay and Wood, 1999; Malyapa et al., 1998; Ostling and Johanson, 1984) and performed by us as described earlier (Golubnitschaja et al., 2003; Moenkemann et al., 2005). For the proper statistical analysis about 200 comets were analysed in a double experiment for each treatment condition and controls.

Western-blot analysis

All analyses were performed twice for each sample. Non-treated (control) and treated glioma cells in non-confluent (day 4 of growth) and confluent (day 9 of growth) cultures were lysed by homogenisation in lysis buffer (9 M urea, Merck, Germany), 1% DTT (Sigma-Aldrich, USA), 2% CHAPS (Merck, Germany), 0.8% Bio-Lyte, pH 3–10 (Bio-Rad, USA), 5 mM Pefabloc (Roche, Switzerland) followed by a centrifugation step. The protein concentration was quantified by the DC-Protein Assay (Bio-Rad, USA). Forty µg protein of each sample were loaded onto 12% SDS-polyacrylamide gels and electrophoresed to separate proteins. The proteins were then transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare, UK) and afterwards incubated at room temperature in blocking-buffer (58 mM $NaHPO_4$, 17 mM NaH_2PO_4, 68 mM NaCl, 5% nonfat dry milk powder; 0.1% Tween 20) for 1 hour. Primary anti-body incubation was performed at room temperature using a 1:250 dilution of the specific anti-bodies (Santa Cruz, USA) in washing buffer I (58 mM $NaHPO_4$, 17 mM NaH_2PO_4, 68 mM NaCl, 1% non-fat dry milk powder, 0.1% Tween 20) for 1 hour. The membranes were then washed four times in the same solution. The horseradish peroxidase-labelled anti-goat secondary anti-body was incubated at room temperature with the membranes in washing buffer I followed by three washes in washing buffer II (58 mM $NaHPO_4$, 17 mM NaH_2PO_4, 68 mM NaCl, 1% nonfat dry milk powder, 0.3% Tween 20) and three washes in washing buffer I. Then the membranes were reacted with chemiluminescent reagent ECL plus (Detection Kit, GE Healthcare, UK) for 1 hour and processed for auto-radiography. The individual signals were measured densitometrically using the "Quantity One" imaging system (Bio-Rad, USA).

Statistical analysis

All statistical evaluations were done using the SPSS software (version 11.0). Overall statistical significance was calculated by ANOVA. Additionally, pairs of datasets were compared by post hoc Turkey's test.

Results

TMZ treatment and combined chemo/radio treatment have highly pronounced DNA damaging effect on malignant glioma cells

In order to qualify damaging effects achieved on chromosomal DNA, cellular comets were classified as follows:

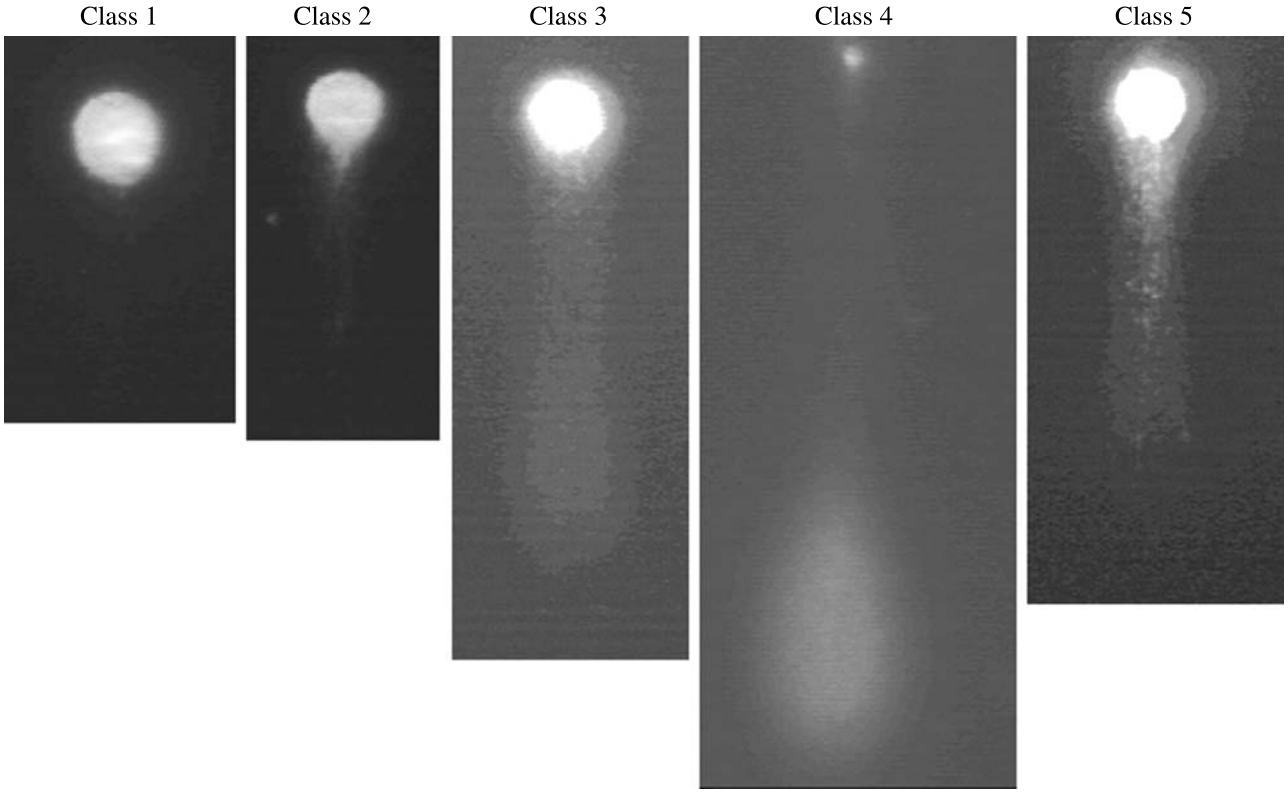

Fig. 1. Comet types classification of DNA in U87MG human malignant glioma cells: class *1* non-proliferative cells with intact DNA; class *2* actively proliferating cells; class *3* non-proliferative cells with partially damaged DNA; class *4* apoptotic cells; class *5* actively proliferating cells with visibly damaged DNA

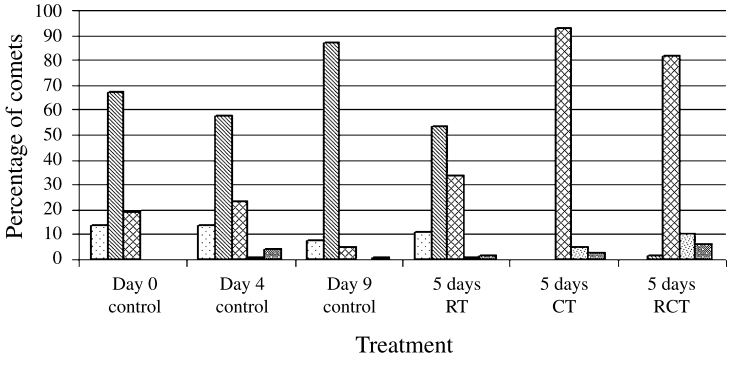

Fig. 2. Comets distribution among five classes (as given in Fig. 1) evaluated for U87MG non-treated (control) and treated cells, where *RT* radiation treatment at day 9th of growth, *CT* chemo treatment at day 9th of growth, and *RCT* chemo/radio treatment at day 9th of growth. ☐ class 1, ▨ class 2, ▩ class 3, ▩ class 4, ▩ class 5

cells with no proliferation (class 1), actively proliferating cells (class 2), non-proliferative cells with partially damaged chromosomal DNA localised within variably long tails of comets which, however, have a well-detectable head (class 3). Furthermore, apoptotic cells with fully damaged DNA localised within a tail of comet, which has no detectable head (class 4), and actively proliferating cells with visibly damaged DNA (class 5) were clearly observed. All these types of comets are shown in Fig. 1. The comets distribution among 5 classes calculated for treated and non-treated cells is shown in Fig. 2. Being monitored immediately after storage at $-80\,^{\circ}C$ (control, day 0), the control pool consisted of three classes of cells only: non-proliferating (class 1, 14%), proliferating (class 2, 67%), and partially damaged (class 3, 19%) cells. There was no significant difference in the distribution of cells among these three classes after 4 days of incubation (control, day 4). Additionally, about 1% of the cells became apoptotic (class 4) and approximately 4% of the cells were shown to be proliferative despite of partial DNA damage (class 3). In contrast, after 9 days of incubation (control, day 9) the absolute majority of cells was

proliferative (class 2, 87%), while the minority was either non-proliferative (class 1, 7.5%) or partially damaged (class 3, about 5%). Compared to controls, the comet distribution was significantly different after all treatments applied during 5 days. After 5 days irradiation applied alone, the comet distribution was similar to that of non-treated cells at day 4 of growth, however, with a 1.5-times increase in class 3 and a 4-times decrease in class 5 comets. After 5 days chemo treatment alone, the absolute majority of cells demonstrated class 3 comets (92%) with no comets of both class 1 and 2 observed, but about 3% of class 5 comets. Similarly, after the combined chemo/irradiation treatment, the absolute majority of cells demonstrated the class 3 comets (82%). In this case, however, 1.3 and 7% proliferating cells were observed in class 2 and 5 comets, respectively.

Not the treatment conditions but a growing cellular density at prolonged cultivation significantly up-regulated P53 and P21$^{waf1/cip1}$ expression levels in glioma cells

The protein expression images for both P53 and P21$^{waf1/cip1}$ are demonstrated in Figs. 3 and 4, respectively. The statistically evaluated expression levels normalised through the corresponding β-actin values are shown in Table 1. Compared to non-treated cells, irradiation and chemo treatment applied alone led to non-significant increase in P53 expression level; the most pronounced effect was shown in the case of combined chemo/radio therapy: in irradiated, TMZ- and chemo/radio treated cells the expression rates were 1.04, 1.07 and 1.64 times higher than those in non-treated cells, respectively. The highest up-regulating effect was observed to be by growing cellular density at prolonged cultivation (1.96-times higher in non-treated cell cultures at day 9 *versus* day 4 of growth).

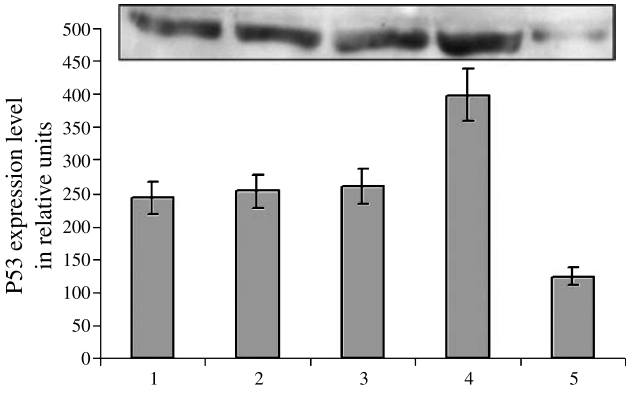

Fig. 3. "Western-Blot" images and evaluation of P53 expression rates in treated *versus* non-treated (control) U87MG malignant glioma cells, where *1* non-treated cells at day 9 of growth; *2*, *3*, *4* treated cells with irradiation, temozolomide, and combined therapy at day 9 of growth; *5* non-treated cells at day 4 of growth. The expression rates of P53 shown in the diagram and in Table 1 were normalised through the corresponding β-actin expression and are given as mean values with standard deviations calculated from parallel experiments

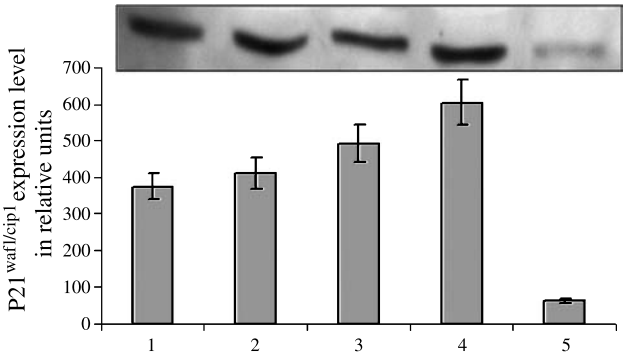

Fig. 4. "Western-Blot" images and evaluation of P21$^{waf1/cip1}$ expression rates in treated *versus* non-treated (control) U87MG malignant glioma cells, where *1* non-treated cells at day 9 of growth; *2*, *3*, *4* treated cells with irradiation, temozolomide, and combined therapy at day 9 of growth; *5* non-treated cells at day 4 of growth. The expression rates of P21$^{waf1/cip1}$ shown in the diagram and in Table 1 were normalised through the corresponding ß-actin expression and are given as mean values with standard deviations calculated from parallel experiments

Table 1. Statistical evaluation of P53 and P21$^{waf1/cip1}$ protein levels in non-treated (control) U87MG glioma cells at days 4 and 9 of growth *versus* treated cells at day 9 of growth after 5 days of daily treatment

Protein	Treatment (day)				
	Control (day 4)	Control (day 9)	RT (day 9)	CT (day 9)	RCT (day 9)
P53	124 ± 10	243 ± 17	253 ± 21	260 ± 25	399 ± 31
P21$^{waf1/cip1}$	62 ± 2	375 ± 32	411 ± 37	492 ± 45	604 ± 29

RT radiation treatment with 2 Gy/day, *CT* treatment with 30 µg/ml temozolomide in cultivation medium, *RCT* combined chemo/radio treatment; all values are given as mean value with the corresponding standard deviation

Similar effects were observed also for P21$^{waf1/cip1}$ expression levels in treated *versus* non-treated cells: in irradiated, TMZ- and chemo/radio-treated cells the expression rates were 1.10, 1.31 and 1.61 times higher than those in non-treated cells, respectively (Table 1). The biggest difference in the expression rates of about 6-times was shown between non-treated (control) glioma cells at day 9 *versus* day 4 of growth, when the cells demonstrated only traces of P21$^{waf1/cip1}$ expression (Fig. 4).

Discussion

Survival of malignant glioma cells under the treatment conditions tested

Nervous system tumours are one of the leading causes of cancer related death. The diffusely infiltrative nature of malignant gliomas is the main obstacle to the successful surgical approach. Current post-operative therapy approaches, however, have minor success as well, since specific mechanisms facilitating the invasive behaviour of gliomas remain obscure. All treatment conditions tested in this work are currently used as the main post-operative therapeutic clinical approaches for patients with malignant gliomas. Our very recent results showed that both TMZ treatment and irradiation applied in clinically relevant doses have sub-lethal effects on malignant U87MG glioma cells (Trog et al., 2006b). Sub-lethal doses of irradiation have been shown to enhance the tumour invasiveness (Hegedus et al., 2004; Wild-Bode et al., 2001). Although resulting in the strongest growth inhibition, the most aggressive combined TMZ/radio treatment leads, however, to not more than 87% cell death. Thus, under each treatment condition tested a considerable surviving cell fraction was observed (Trog et al., 2005). This conclusion correlates well with the clinical outcome, since tumour reoccurrence is usually observed in malignant gliomas after radio or/and chemo treatment.

DNA damage and cell cycle control

"Comet assay" results showed an extensive DNA damage caused particularly by TMZ treatment applied alone and in combination with irradiation, correlating well with the smallest cell fractions survived under these treatment conditions (Trog et al., 2005). In contrast, irradiation resulted in a relatively low DNA damage correlating well with a considerable surviving cell fraction and indicating a poor therapeutic efficiency of irradiation applied alone. Noteworthy, malignant glioma cells surviving after TMZ treatment demonstrated a tremendous amount of non-repaired DNA that consequently gives rise to variable mutations. Remaining non-repaired in proliferating glioma cells – qualified as class 5 in "Comet assay" (Fig. 1) – these mutations might, further, increase the malignancy grade in following cell generation. This conclusion is supported by the clinical observation: compared to the original gliomas, the reoccurred ones are frequently more aggressive and carry multiple mutations in tumour suppressor genes (Ohgaki et al., 2004).

U87MG cell line demonstrates relatively low P53 expression levels compared to other malignant glioma cells, and the abrogation of wild-type *p53* function by RNA interference significantly protects U87MG glioma cells against TMZ treatment (Hermisson et al., 2006). Our results achieved in this work considerably furthered this important knowledge: under genotoxic therapeutic treatments tested here, only minimal up-regulating effect in the expression patterns of P53 was observed. In contrast, rapid significant up-regulation of P53 expression levels has been shown to be an adequate response of normal mammalian tissues to DNA damaging conditions (Golubnitschaja et al., 2003; Golubnitschaja-Labudova et al., 2000; Moenkemann et al., 2005). Obviously, the low expression level of P53 might be an important characteristic feature of U87MG glioma cells, due to which these malignant cells can survive particularly under TZM and radiation treatment. In agreement with this conclusion, other studies showed that only very few of TMZ treated U87MG glioma cells undergo apoptosis, whereas the majority of them undergo senescence over a 10-day period of treatment (Hirose et al., 2001). Our results also support the dominant role of senescence in cell cycle regulation of U87MG glioma: the prolonged cultivation of non-treated cells is 1.5- till 5-times as strong activator of both P53 and $P21^{waf1/cip1}$ as genotoxic TMZ and radiation treatments. Furthermore, the growing body of evidence proposes the enhanced expression levels of $P21^{waf1/cip1}$ to be an attribute of senescence progression (Chang et al., 2000). Therefore, we conclude here that senescence plays in cell cycle regulation of U87MG glioma more important role than genotoxic conditions created by both TMZ and radiation treatment.

In consensus to the P53 expression regulation observed, similarly low up-regulating effect was monitored also for the $P21^{waf1/cip1}$ expression under the treatment conditions tested. The cdk inhibitor $P21^{waf1/cip1}$ – the downstream effector of and transcriptionally activated by wild-type *p53* – functions as the check point protein to block cell cycle progression in the G_1 phase. In experiments with *p53*-defective U-373 MG human astrocytoma and T-98G glioma cell lines, it has been shown that an enhanced expression of $P21^{waf1/cip1}$ inhibits proliferation, tumourigenicity and aneuploidy, and induces cell differentiation of malignant gliomas (Chen et al., 1996; Kokunai et al., 1998; Kominsky et al., 1998). Our results indicate that not a lack of expression, but the deficits in response to genotoxic conditions in terms of P53 and $P21^{waf1/cip1}$ expression regulation may play the crucial role in the resistance towards pro-apoptotic stimuli, uncontrolled cell growth

and survival of malignant gliomas under TMZ or/and radiation treatment. In contrast, both P53 and $P21^{waf1/cip1}$ were found to be significantly up-regulated by high cell density at prolonged cultivation. The well-known specific feature of malignant gliomas is an obligatory dependence on the development of its own vascular system for adequate oxygen and nutrient delivery. Thus, malignant glioma cells of high density induce their own vasculature formation (Ader et al., 2003). We speculate, therefore, that the cell cycle control in glioma cells might have a function which differs from maintaining of the original phenotype of single cells: cell cycle attributing proteins are activated in concert with pro-angiogenic events, when the tissue becomes remodelled and the processes of neovascularisation are triggered (Trog et al., 2006b).

Concluding remarks

Taken together, this work considers potential molecular mechanisms which may play a crucial role in the resistance of malignant glioma cells towards clinically relevant treatment conditions. Our results show that not one kind of treatment tested is able to suppress glioma cell proliferation completely. Moreover, ''Comet assay'' imaging shows clear proliferation among glioma cells with strongly damaged DNA, indicating the way how malignancy grad can be promoted in the following generation of treated glioma cells. Noteworthy, not the stress conditions tested but a growing cellular density and prolonged culturing of non-treated cells significantly up-regulate both P53 and $P21^{waf1/cip1}$ expression levels in glioma cells. This indicates 1. a non-sufficient cell cycle control under genotoxic conditions and 2. an essential role of both proteins in tissue organising of high density malignant glioma cells.

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