Journal of Neuro-Oncology (2005) 73: 9–18
DOI 10.1007/s11060-004-3341-3

Laboratory Investigation

The brain slice chamber, a novel variation of the Boyden Chamber Assay, allows time-dependent quantification of glioma invasion into mammalian brain *in vitro*

Christian Schichor[1], Siglinde Kerkau[2], Theresa Visted[3], Rudolf Martini[4], Rolf Bjerkvig[3], Jörg Christian Tonn[1] and Roland Goldbrunner[1]
[1]*Department of Neurosurgery, University of Munich, Germany;* [2]*Department of Neurosurgery, University of Wuerzburg, Germany;* [3]*Department of Anatomy and Cell Biology, University of Bergen, Norway;*
[4]*Department of Neurology, University of Wuerzburg, Germany*

Key words: glioblastoma, glioma, invasion, *in vitro*, model, paclitaxel; vincristin

Summary

Glioma cell invasion occurs in a complex micromilieu consisting of neural and glial cells, myelinated fiber tracts, blood vessels and extracellular matrix proteins. The present work describes the brain slice chamber (BSC) as a novel experimental model for assessing invasion of glioma cells into adult mammalian white and gray matter on the basis of the well known Boyden chamber system. As a matrix for invasive tumor cells we used freshly prepared brain tissue from adult pigs. The tissue was sectioned into 40 μm slices that were mechanically fixed to a millipore filter. The neural structures and the three-dimensional architecture of the slice was preserved as verified by immunohistochemistry, light- and electron microscopy. Human U-373 and U87 astrocytoma cells stably transfected with green fluorescent protein (GFP) were assessed for their invasiveness into the brain-slices during a 24 h period. Invasion of U-87 GFP cells was quantified at different time intervals by confocal laser scanning microscopy showing more intense invasion into white compared to gray matter. Two cytostatics (vincristin and paclitaxel) which both are known to affect the cytoskeleton, inhibited glioma cell invasion in a dose dependent manner, which makes the presented model system suitable for functional experiments. In conclusion, the BSC represents a valid and rapid experimental model that may be used to describe the invasive behavior of glioma cells within the preserved three-dimensional structure of mammalian brain tissue *in vitro*.

Introduction

The normal brain and gliomas themselves contribute to an unique biological microenvironment which leads to an extensive dissemination of tumor cells into normal brain structures [1]. Several *in vitro* models have been developed to study the cells' invasive behavior into artificial substrates. As an invasion matrix, gels consisting of single proteins (collagen, laminin) as well as protein mixtures have been used. One of the most widely used substrates is Matrigel™ that has been used in a double chamber system (Boyden Chamber Assay) [2,3].

Even though Matrigel™ consists of some of the main constituents of the extracellular matrix (e.g. Laminin, Collagen IV and Entactin), Matrigel™ lacks cellular components as myelin sheaths as well as a three-dimensional architecture of the white matter, which represent the major substrate for the invading tumor cells.

Glioma invasion is the result of a complex interaction of individual cells with surrounding structures of the brain. The tumor cells interact with the environment by means of cell surface receptors and by producing specific proteases. Thus, cell behavior that can be observed in a reconstituted matrix is possibly influenced by the cell's response to this artificial environment. The aim of the present work was to develop a novel experimental model to study glioma invasion *in vitro* minimizing this artificial factor. The assay should combine the advantages of the Boyden Chamber Assay (e.g. easy handling, use of single cells) with the use of a physiological mammalian brain matrix, reflecting its three-dimensional cellular and extracellular architecture. In addition, this assay should provide quantitative results as well as morphological data of the interaction between glioma cells and white matter *in vitro*. Thus, a combination of the Boyden chamber and an organotypic co-culture system was developed placing slices of white and gray matter of porcine brain [4] on top of a filter in a double chamber system.

Materials and methods

Brain slices

Frontal and parietal lobes of the domestic pig was prepared under sterile conditions, immediately after sacrificing the animal. The material was cut into blocks, measuring $0.5 \times 0.5 \times 0.5$ cm and snap frozen at -70 °C. During preparation, subpial and subarachnoidal spaces

were dissected to avoid areas where passive cell distribution could occur. Slices were sectioned (10, 20, and 40 μm) at −20 °C respecting areas of white and gray matter. Neurons were visualized with Nissl staining, white matter myelin was stained with Luxul Fast Blue staining, axons were labeled by Bodian silver impregnation and immunostaining for neurofilaments (murine monoclonal; clone 2F, dilution 1:100; DAKO, Hamburg, Germany). Astroglial cells were stained with rabbit anti-cow glial fibrillary acidic protein (GFAP) anti-serum (DAKO) in a dilution of 1:4000. Immunostainings were carried out on a Techmate 500 immunostainer (DAKO) using the ABC-Peroxidase method according to the manufacturer's description. The brain tissue was freshly prepared for the experiments (90 min), but also at later time points (3 h, 4 h, 5 h, 8 h, 12 h) after the pig's death in order to investigate histological changes of the tissue after circulatory arrest. For invasion experiments, only fresh slices (less than 90 min after the pig's death) were used.

Cell lines

The human glioma cells lines U-87 and U-373 (purchased from American Type Culture Collection, ATCC; Manassas, VA) as well as green fluorescent protein (GFP) transfected U-87 cells (U87GFP) were used. Monolayer cultures were grown in 75 cm^2 tissue culture flask (Nunc, Roskilde, Denmark) in a standard tissue culture incubator (5% CO_2 in air, 100% humidity). The cells were grown in complete growth medium consisting of Dulbecco's Modified Eagle's Medium supplemented with 10% newborn calf serum, four times the prescribed concentration of non-essential amino acids, 4 mM L-glutamin, penicillin (100 IU/ml) and streptomycin (100 μg/ml) (all reagents: BioWhittaker, Verviers, Belgium). The U-87GFP cells were routinely propagated under the same conditions with an addition of 1.2 mg/ml G-418 (Sigma, St. Louis, MO) to the medium.

GFP transfection

The transfection vector pEGFP-N1 was purchased from Intermedica (Stockholm, Sweden). The vector expresses enhanced GFP, a redshifted variant of wild type GFP of the jellyfish Aequorea victoria (with excitation maximum 488 nm; emission maximum 507 nm). 5×10^5 U-87 cells were plated in 25 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark). GFP liposome transfection was performed with Lipofectin (GibcoBRL, Life Technologies, Paisley, Scotland) according to standard procedures.

Preparation of the brain slice chamber (BSC)

The lower wells of the BSC (Costar™, Sigma-Aldrich, St. Louis, MO) were filled with 100 μl of medium, millipore filters (pore size 8 μm) were inserted, and the whole assembly was placed into the cryomicrotome (−20 °C). This was done in order to level out temperatures of the brain slices and the chamber. The upper wells (screw) were kept at room temperature. Slices (area 18 mm^2, thickness 40 μm) of white or gray matter were placed on top of the filter with a cooled forceps (−20 °C) within the cryostat. To compare invasion into different anatomical structures we used both, slices (40 μm) of gray matter (parietal cortex) and frontal white matter. The lower wells (−20 °C) were closed with the screw. In order to avoid damaging the cells by the temperature of the chamber, 100 μl of complete growth medium at 37 °C was added to the upper well. Five minutes later, single-cell suspensions of U-373, U-87 or U-87GFP cells were added in a total volume of 200 μl complete growth medium for assessment of invasion. See Figure 1 for complete experimental setup.

Invasion assay

1×10^4 or 2×10^4 U87-GFP cells were placed into the upper well. The BSC was kept in a standard tissue culture incubator (5% CO_2 in air, 100% humidity) for 24 or 48 h. Invading cells were visualized by light, electron- and confocal microscopy (see below). Intensity and velocity of glioma cell invasion was calculated by correlation of GFP fluorescence intensity with cell number at different depths within the tissue and at different time points. Glioma cells treated with 98% ethanol served as negative controls.

Generation of semi- and ultrathin sections for light- and electron microscopy

For conventional light microscopy, the filter covered by the invaded brain slice was washed in PBS in order to remove unattached cells from the slice. Thereafter, the brain slice were stained according to standard procedures (Papanicolaou: single agent staining for cell visualization) and mounted on glass. For electron microscopy, the cultures were fixed by immersion into cacodylate buffer containing 4% freshly depolymerized paraformaldehyde and 2% glutaraldehyde for 6 h at room temperature, followed by osmification in 2% OsO_4 for 2 h. Then cultures were dehydrated in ascending series of acetone and embedded in Spurr's medium. Embedded cultures were trimmed and either semithin or ultrathin sections were prepared. Semithin sections were used for light microscopy, stained with basic methylene blue and investigated in a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany). Ultrathin sections were counterstained with lead citrate (Reynolds) and investigated with a Zeiss EM 10B electron microscope (Zeiss, Oberkochen, Germany).

Confocal microscopy

The filter and the brain slice were washed in PBS, transferred to a cover glass and covered by a small volume of PBS to avoid drying of the brain slice. Experiments were performed using a confocal laser scanning microscope (Leica TCS NT, Leica Lasertech-

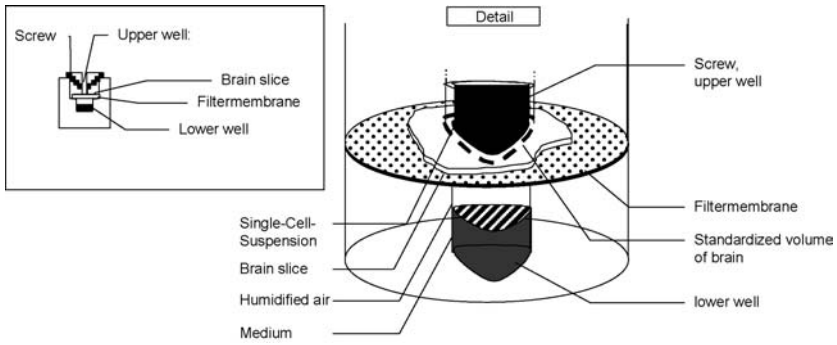

Figure 1. Arrangement of the BSC.

nik GmbH, Heidelberg, Germany) attached to an upright microscope (Leica DM RXA). For the optical sectioning a 16 × objective of Leica was used. Distilled water was used as immersion medium. The optical sections were recorded and quantified with the native Leica software (version 1.6.587). Typically, 32 optical sections with a resolution of 512 × 512 per channel were recorded with an appropriate step size. Up to 16 pictures were averaged in order to improve the quality using the slow scan mode. Every slice was analyzed in 4 different areas. FITC was excited with the 488 nm line of an Ar–Kr laser and the fluorescence was detected using a band pass filter. The pinhole settings were optimized gaining maximal confocal quality. Details on confocal laser image processing have been described elsewhere [5]. For quantitative analysis of differential invasion into white and gray matter, cell counting of confocal images was performed using eight sections of gray and white matter by two observers in a blinded way. Statistical analysis was done using student's *t*-test with $P < 0.01$.

Drug treatment protocol

The invasion assay described above was performed using 2×10^4 cells for a time period of 24 h. Cells were treated by adding increasing concentrations of paclitaxel (Taxol®, 0.05 μg/ml, 0.1 μg/ml, 0.2 μg/ml, 0.5 μg/ml, purchased from Bristol/Myers/Squibb, Munich, Germany) and vincristin (5 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml) to the upper well of the BSC at the beginning of the experiment. To exclude cytotoxicity by the drug concentrations used, U87 monolayer cells were treated by the same concentration and their viability was assessed by light microscopy.

Results

Analysis of the invasion substrate

Cellular composition, axon density and myelination of the fresh brain slices showed no pathological alterations. An increased number of astrocytes and astrocytic processes was not detected adjacent to vessels in the white matter indicating the absence of pathological reactive gliosis. The neuronal cells present in some areas of the slide showed a normal cytoarchitecture without any signs of neuronal damage (Figure 2a, c).

Histological investigations of pig brain tissue 90 min, 3 h, 4 h, 5 h, 8 h, and 12 h after the pig's death revealed eosinic changes of neurons and pericarial swelling, mainly in the white matter, first seen after 3 h, but more obvious at later time points. At 90 min, GFAP staining of the tissue showed single, predominantly perivascular astroglial cells, exclusively in the white matter. At 3 and 4 h there was increased staining of reactive astrocytes in the white matter tissue, resulting in a strong astroglial reactivity 5 h after death of the pig. Density of reactive astrocytes of the white matter increased after 8 and 12 h. In addition, there were also astrocytes labeled in subpial areas. There were no gross abnormalities of axons as judged by Neurofilament and S-100 protein staining, Bodian silver impregnation and Luxul fast blue staining (Data not shown). The electron microscopic analysis of the cryosections revealed a good preservation of the neural structures. Axonal profiles, astrocytic processes and cell somata of glial or neuronal cells could be identified. From the various neural structures, myelin sheaths were best preserved, particularly in the center of the cryosections. These findings are in concordance with previous observations of our group in cryosections of peripheral nerves, where myelin sheaths were best preserved as well [67].

Optimization of the experimental setup

The schematic setup of the BSC is depicted in Figure 1. According to the above-mentioned procedures the brain slices were–without any fixatives – optimally preserved during the time period studied. To achieve this, we investigated several variations of the setup.

Lower well content

The lower well of the BSC (total volume: 200 μl) was filled with 100 μl of DMEM in order to keep the brain slice at a borderline between fluid and (humidified) air. This borderline was maintained due to the filter's pore size (8 μm) which prevented medium to run from the upper well into the lower. If the lower well was filled up with 200 μl of medium the brain slice came into contact with fluid from both sides and was dissolved. Without any fluid in the lower well, the structures of the brain slice were not preserved as well as with a small volume (100 μl)

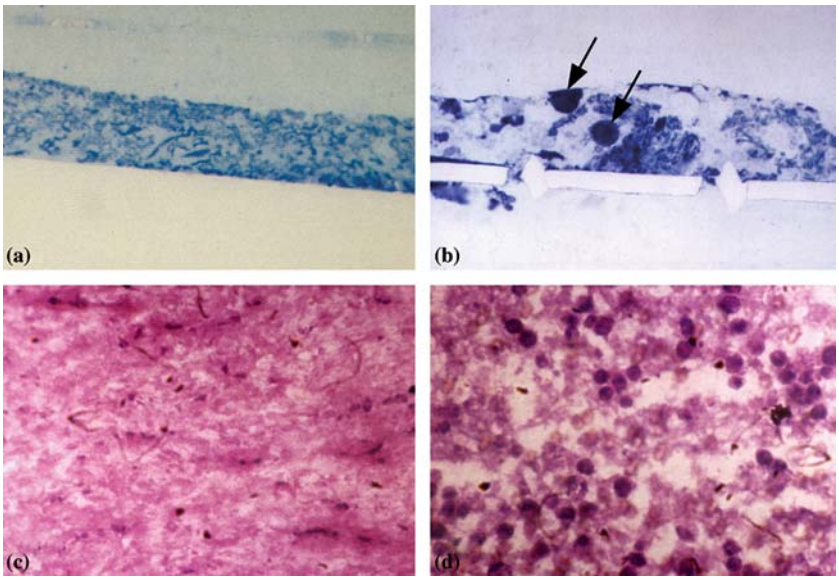

Figure 2. (a) Semithin cross section of a fresh brain slice 1 h after sectioning, stained with Luxul fast blue. Magnification 400×. (b) Semithin section of a fresh brain slice during glioma cell (arrow) invasion depicting destruction of brain tissue surrounding glioma cells, magnification 500×. (c) Section of a fresh brain slice stained according to the Papanicolaou protocol, magnification 400×. (d) Tumor cell infiltration of the brain slice (Papanicolaou staining), magnification 400×.

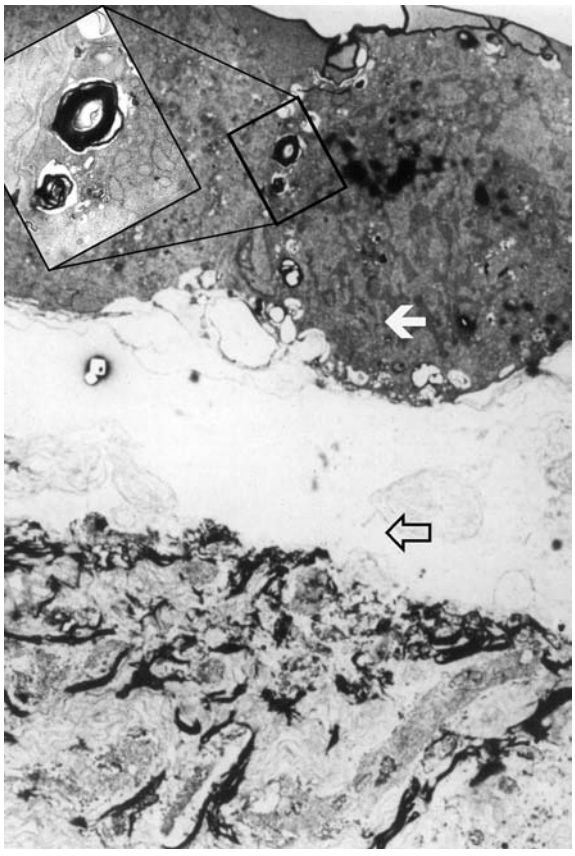

Figure 3. Electron microscopic imaging of semithin sections showing glioma cells in close contact to nerve sheaths. Around glioma cells (white arrow) large areas of dissolved tissue (open arrow) without any inner structure is seen. Insert upper left corner: magnified area showing myelin sheaths at the interface between two glioma cells.

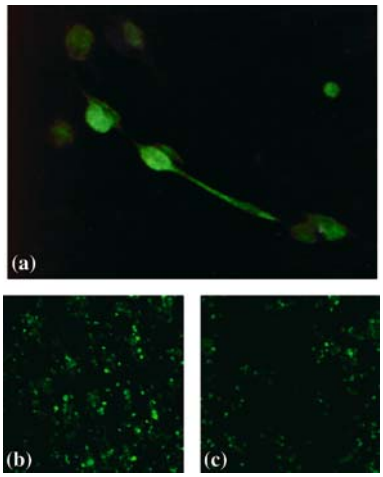

Figure 4. (a) U87-GFP cells during invasion along a white matter tract of the brain slice. They display long processes paralleling the fiber tracts of the slice. (b) Invasion into white matter. (c) Invasion into gray matter. Quantitative comparison of B and C reveals a more than two times stronger preference for white matter invasion (significant with $P < 0.01$).

of fluid, which provided some humidity to the tissue during the incubation period. The type of fluid added to the lower well, either a chemoattractant (3T3-conditioned medium) or DMEM, did not affect invasion rates (data not shown).

BSC temperature

In order to place the slice in the center of the filter properly, it was essential to keep the temperature of the chamber at −20 °C similar to that of the slice. The slice could easily be removed from the blade of the cryostat with a cooled forceps (−20 °C) and transferred to the chamber which was kept in the cryostat as well. Cooling of the chamber or the filter did not affect their shape or appearance.

BSC closure

After placing the slice on the filter, the chamber was closed with the screw (the upper well), which was kept at room temperature. Due to the different temperatures, the warm screw closed the cooled chamber tightly. The screw was adding a very light pressure to the slice and kept it in the correct position. Without the screw, the slice was washed off. If the temperatures of screw and lower well were similar (−20 °C or room temperature), the brain slice was damaged by the lower rim of the screw due to a too strong closure. After the closure of the chamber, 100 µl complete growth medium (37 °C) was added in order to adjust the temperature before adding the cells.

Invasion into the brain slice

After 12 h, glioma cells had invaded the slice but could not be seen light-microscopically from its lower side. After 24 h, first single glioma cells were detected at the lower side of the 40 µm brain slice. By increasing number of cells (10,000 to 40,000 cells) in the upper wells, more invading cells were seen invading the brain tissue. The invading tumor cells could only be detected within the brain-tissue, not on the lower side of the filter or in the medium of the lower well. Invasion occurred in a rather homogenous way (Figure 2b,d).

Morphological analysis

Light microscopic investigation of semi-thin sectioned slices without any cells (controls) displayed that surface and shape of the slice had been maintained after 48 h. Slices exposed to invading tumor-cells showed cells close to the surface as well as deep within the slice after 24 h. The structure of the slice was destroyed in close vicinity of the invading cells (Figures 2b and 3). These findings were verified by electron microscopic imaging, which demonstrated areas of dissolved tissue around glioma cells, which commonly are in close contact to nerve sheaths (Figure 3). Confocal microscopy could easily detect U-87GFP cells within the non-fluorescent brain-slice. They displayed a regular and round shape of the cell body with multiple long parallel processes along parallel and intersecting fibers of the slice (Figure 4a). Comparing invasion of U87-GFP cells into white and gray matter, a much more intense invasion of white matter was detected than into parietal gray matter (Figure 4b,c).

Quantitative analysis

Intensity signals of GFP-transfected cells can be measured under the confocal microscope. Increasing cell counts are reflected by an increasing intensity signal. By detecting cells throughout the three dimensional slice stepwise from its top to the bottom, the total cell count could be estimated. The maximum signal intensity could be located at a certain depth within the slice, thus reflecting the depth of the peak of the invading cells. Increasing cell numbers added to the upper well correlated with an increasing amount of invading cells detected within the tissue signal. In contrast, the depth of invasion during 24 h was not affected by the two different amounts of cells added to the upper well. Both cell numbers, 1×10^4 as well as 2×10^4, reached a comparable depth of invasion (Figure 5). The maximal cell count could be detected at 30 µm in the brain slice after 24 h with either cell amount. Regarding the different invasion into gray and white matter, cells were counted in eight representative sections each in a blinded way. This revealed 30.6 ± 8.7 cells per field in white matter whereas 14.1 ± 9.2 cells per field were counted in gray matter (Figure 4b,c) indicating a statistically significant difference ($P < 0.01$).

Velocity of invasion

The depth of invasion was related to the time of incubation. Constant initial cell counts of 20.000 cells per well were used. After 12 h incubation, cells only could be detected close to the surface within the tissue. After 18 and 24 h, the depth of invasion and the amount of cells within the tissue (the maximum intensity) increased. After 48 h many cells had reached the lower side of the brain slice (Figure 6). Hence, 0.8 µm/h can be calculated as maximum speed of invasion in our model. An increased intensity signal all over the depth (40 µm) of the slice indicated fluorescent cells at different levels within the tissue, showing a different invasive potential of the individual cells. No fluorescent glioma-cells could be detected within the medium of the lower well.

As a negative control, cells were treated with 98% ethanol. They could not be detected within the brain slice excluding any passive cell distribution within the slice.

Inhibition of invasion

In order to test the ability of the BSC for functional assay, we added substances which are known to inhibit invasion and migration of glioma cells. Treatment with anti-invasive drugs (vincristin, paclitaxel), which affect the cytoskeleton of the cells [8–10], reduced invasion in a dose-dependent manner. Direct cytotoxity by the concentrations used could be excluded in U373 and U87 monolayers assays where cell viability was not affected. In the BSC, at 10 ng/ml vincristine no effect on U373 invasion over 24 h could be seen by light microscopy. Increasing concentrations of vincristine resulted in a dose-dependent reduction of cells counted in the tissue. At 100 ng/ml vincristine the invasive process was inhibited completely. The same dose dependent inhibition of invasion was observed using paclitaxel. This process was also quantified by the measurement of the intensity of the GFP-transfected U87 glioma cells by confocal microscopy. Using 0.05 µg/ml there was only little effect compared to the untreated controls. With increasing paclitaxel concentrations the amount of invading cells, as well as the depth they reached, was reduced (Figure 7). At 0.5 µg/ml, the total number of invading cells at the peak depth was reduced to 38% and the peak depth was decreased by 60% compared to untreated controls after 24 h.

Discussion

Glioma cell invasion can not only be seen as a proteolytic destruction of tissue and random movement of the cells. It is the result of a complex interaction of the tumor with the surrounding brain parenchyma. Receptor-dependent adhesion and subsequent expression of specific proteases which degrade proteins of the extracellular matrix lead to migration along anatomical structures [11,12]. These structures include the basement membrane of blood vessels, the subependymal space, the glia limitans externa and parallel or intersecting nerve fiber tracts of the white matter. They contain a variety of proteins which serve as a preferred substrate of invasion, mainly laminin, collagen IV, tenascin and hyaluronic acid. Additionally, the vascular basement membrane consists of heparan sulfate, proteoglycans, collagen I, fibronectin, vitronectin and entactin. The ECM of the gray matter is mainly composed by glycosaminoglycans like hyaluronic acid, chondroitin sulfate, keratin sulfate, heparan sulfate and few structural ECM glycoproteins [13–16]. There is a large variety of specific cell-matrix adhesion molecules (the superfamily of integrins) expressed at the surface of invading glioma cells as well as receptors that mediate adhesion of cell to cellular components (cadherins, selectins, immunoglobulines like neural cell adhesion molecules (NCAM), intercellular cell adhesion molecules (ICAM) and vascular cell adhesion molecules(VCAM)) [14]. These extracellular components are presented to invading glioma cells not as a homogenous gel, but they are integrated into a complex three-dimensional architecture which influences the migratory behavior [14–17].

In order to study the multifaceted process of invasion, glioma cells have been exposed to different invasion matrices *in vitro*. Two- and three-dimensional models have been used, cells have been implanted as cell suspensions or spheroids into collagen wafers and other gels, consisting of single ECM components [18–21] Additionally, mixtures of different ECM-constituents have been employed such as the so-called 'reconstituted basement membrane' (Matrigel™), which is widely used in the Boyden Chamber assay. Matrigel™ is produced by the Engelbreth-Holm-Swarm sarcoma, which is of murine origin. It is a homogenous mixture of mainly two separated networks of laminin and collagen IV, which are connected by bridging heparan sulfate proteoglycanes and entactin. Matrigel™ represents–in part–some of the main components of the human brain ECM. As it was thought to be a reconstituted basement membrane, some authors have been able to correlate the invasive behavior of human metastasizing tumors in Matrigel™ with their capability to traverse basement membranes as an initial step to metastasis *in vivo* [22,23]. However, Matrigel™ not only lacks some of the above mentioned constituents as well as cellular components and myelin, but also does not reflect the white matter's three-dimensional structure; the latter being a major factor determining glioma cell motility *in situ*. Aim of this study was to find a novel *in vitro* model for glioma cell invasion using a matrix which shares a maximum of characteristics with the invasion matrix *in vivo*. Therefore, the preparation of mature white matter as well as gray matter was thought to provide a possibility to study the invasive behavior of glioma cells *in vitro* by the use of a microenvironment derived from the invasion substrate *in vivo*. On the other hand, the assay should be able to be standardized in order to analyze factors influencing invasion.

In the BSC, invading cells are analyzed *within* their invasion substrate, the brain slice. In other experimental setups, like the Boyden Chamber assay, there are differing ways of analyzing the invasive process. The cells are first invading the Matrigel™-layer, then they adhere to the porous membrane, migrate to a pore, traverse the filter and adhere to its lower side, which contacts the content of the lower well. Some authors count the cells within the Matrigel™ [24], others take away the Matrigel™ with a cotton swab and count the cells, which adhered to the lower side of the filter [22], and some authors count the cells that fell into the lower well [18]. The most widely used method is to count the cells which adhered to the lower side of the filter. However, invasion in Matrigel and traversing of the filter-pores have been

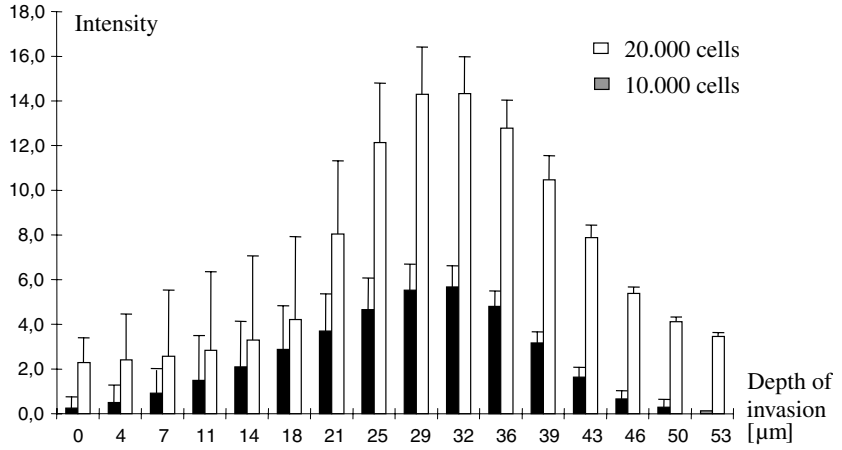

Figure 5. Quantification of depth of invasion [µm] after 24 h by confocal laser scanning microscopy. Maximum GFP fluorescence intensity signal of both cell counts, 1×10^4 and 2×10^4, is detectable at 30 µm within the brain slice. Four slices have been analyzed at four different sites each. Values are given as means, bars represent standard deviation, $n = 16$.

shown as two separate actions of the moving cell which are not correlated to each other [23]. Additionally, in the Boyden Chamber Assay, chemoattractants in the lower well are widely used to increase the invasion rate. However, the use of stimulating chemoattractants affects cellular invasion mechanisms. This artifact can be avoided in our chemoattractant free assay.

A major issue in our setup was fast preparation of the brain slice after the pig's death to avoid substantial damage to the myelinated fiber tracts, since preservation of the myelinated structures under culture conditions was essential for our model. A long time interval between the individual's death and the use of the white matter in cell adhesion assays with subsequent disinte-

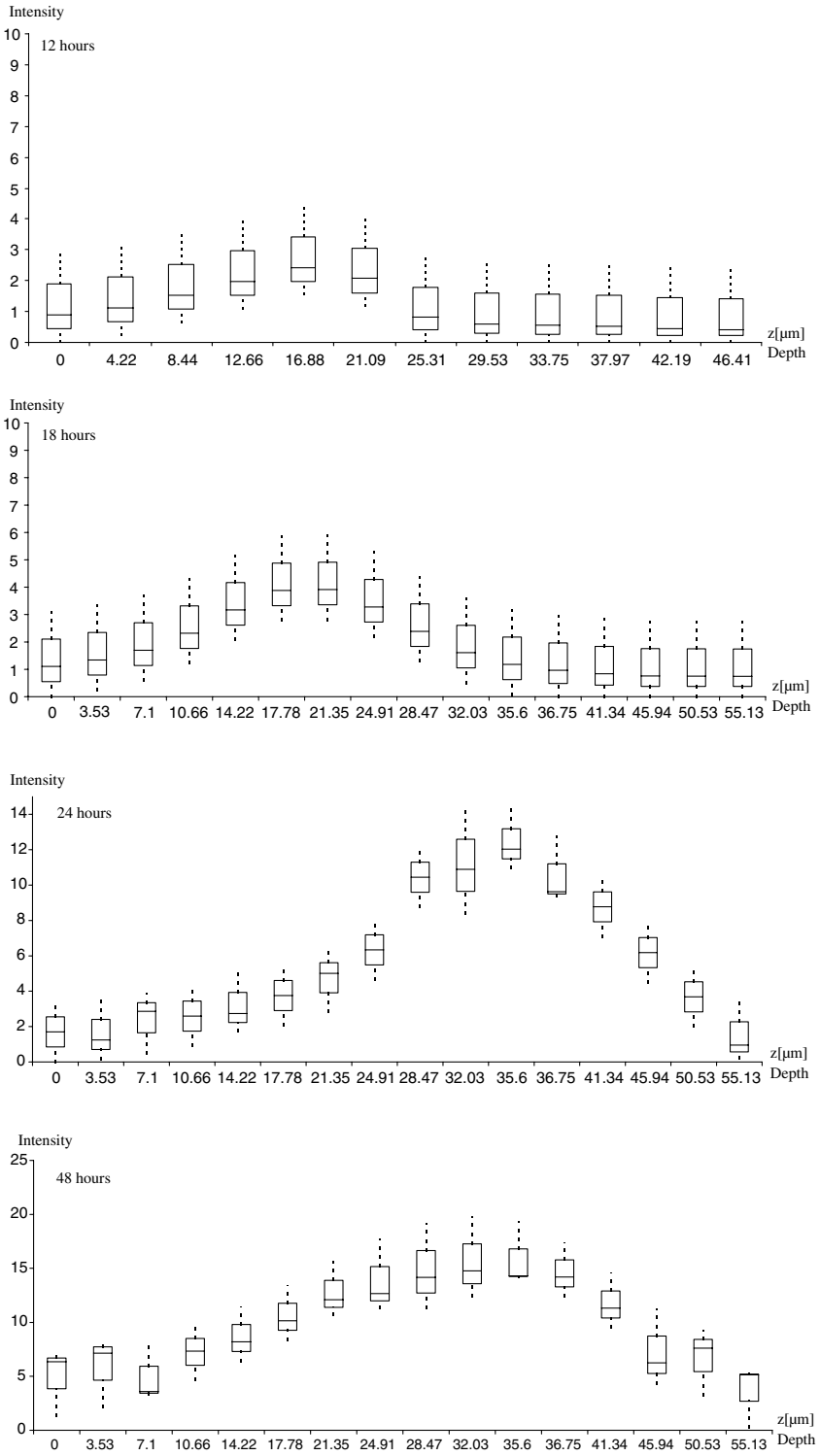

Figure 6. Quantification of depth of invasion after 12, 18, 24 and 48 h by confocal laser scanning microscopy. Initial cell counts were constantly 20,000 per well. Depth of invasion as well as cell counts within the tissue increased by time. After 48 h the majority of cells had reached the lower side of the brain slice. Inhomogeneous distribution of signal intensity shows a different invasive potentials of the individual cells. Four slices have been analyzed at four different sites each. Values are given as box-and-whisker blots. Mean: center of the boxes, 1.-3. quartile: borders of the boxes, extremes: single dots, $n = 16$.

gration of the myelin could account for the observation of reduced adhesion and invasion of glioma cells in white matter compared to gray matter [25], an observation that was contradictory to former pathoanatomical experience [26]. This is the first time that an *in vitro* assay is presented which preserves the white matter in a way that it is more invaded than gray matter. It reflects the clinical behavior of gliomas, as these tumors rarely invade the human cortex, but extensively white matter tracts [14–17].

The most important feature for the preservation of the slice even for a longer time period (up to 60 h) was to keep the slice at a surface between medium and air. This method is well established for neurophysiological experiments with organotypic cultures and provides the potential to preserve slices of brain for up to 70 h [27,28]. But in contrast to our setup, in neurophysiological assays the air is on the upper side of the brain slice which covers a filter, swimming in the medium. We tilted this air-medium-border upside-down in order to have the opportunity to add cells to an upper well filled with medium. The pore size of the filter was big enough to allow the humidified air from the lower well to reach the covering brain slice on top. The constant volume of air transmitted through the pores is an immanent feature of the filter and its pores' size (8 μm). By this technique we avoided to fix the brain slice to the filter with any fixative agents, which, by themselves, could affect the invasive behavior of the cells.

Since fresh human brain is rarely available for routine *in vitro* cultures, porcine brain was used as a substrate for invasion in the BSC assay. As the size of the exposed area in this setup is 18 mm^2, porcine brain is big enough to provide large amounts of homogenous pieces of white

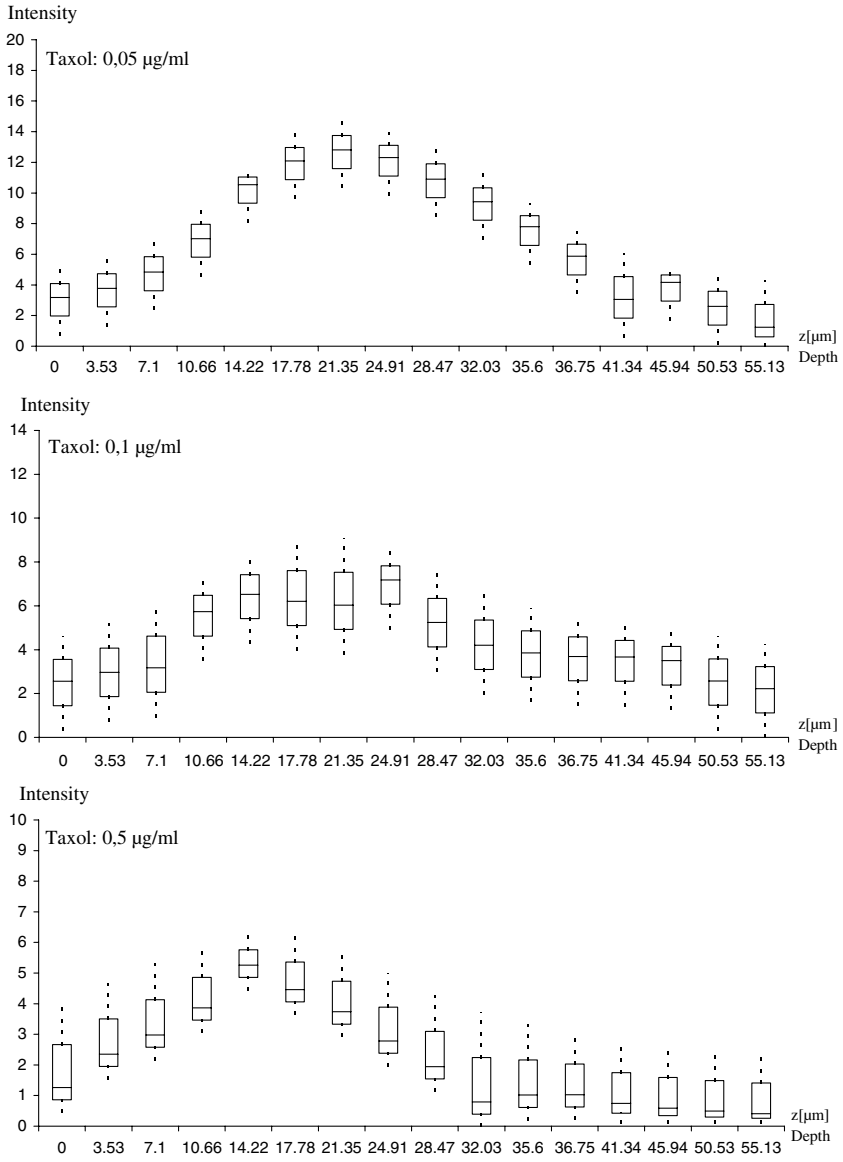

Figure 7. Quantification of depth of invasion after treatment with increasing concentrations of paclitaxel. A dose dependent reduction of invading U87 cell number as well as invasion was found. At 0.5 μg/ml the number of invading cells at within the peak depth was reduced by 62% and a decrease of the peak depth by 60% was seen. Evaluation was performed according to the maximum intensity signal. Four slices have been analyzed at four different sites each. Values are given as box-and-whisker blots. Mean: center of the boxes, 1.-3. quartile: borders of the boxes, extremes: single dots, $n = 16$.

or gray matter. The brain anatomy of the pig as a mammalian is comparable to the human brain, its relationship of white and gray matter-volume is more closely related to the human brain than for example the rat's brain, which contains relatively more gray matter [29,30]. The significance of differentiation between white and gray matter invasion has been emphasized by the fact that white matter invasion was found two times more often than gray matter invasion. This is an important difference to other brain slice models which utilize rodent brain [31–33]. Microglial cells have been isolated from pig's brains and molecular analysis showed more common elements of human and porcine brain microglia compared to murine brain [4]. Pathological anatomy also shows similarities as there are porcine astrocytic tumors as well [34,35]. For these reasons, the use of mammalian brain, particularly porcine brain, is absolutely preferably compared to murine or other brain specimens.

Similar to the BSC model, other brain slice models assessing glioma cell invasion have been described recently: Yoshida et al. [33] presented a brain slice assay using whole murine brain. The problems with using rodent brain have been discussed above. In this assay, cells were added into an artificial pinhole of the slice as a Matrigel embedded tumor spheroid. In contrast to the BSC assay, this assay is restricted to visualization of fluorescent cells and is compromised by addition of Matrigel as an additional artificial factor. The multidirectional cell invasion observed in that model has been avoided in the BSC model, where unidirectional invasion allows a clear quantitative analysis. Jung et al. [36] described an organotypic brain slice culture utilizing human brain. In this assay, fluorescent cells were positioned in a hole of a brain slice, which was swimming on a membrane with medium below. Also in this setting, only fluorescent cells could be visualized within the slice in contrast to the BSC assay, where fluorescent as well as non-fluorescent cells can be quantified either by counting in the light microscope or by confocal microscopic quantification. Furthermore, in the BSC medium is on the upper side of the slice in a way experimental substances can be added easily. Jung et al. measured invasion up to 10 days, what is not possible in the BSC. The BSC was designed as a short term model to exclude a possible overlap of invasion and proliferation.

In order to investigate external influence on active movement of cells within the matrix in the BSC, increasing concentrations of two different well established inhibitors of migration were added. Vincristine and paclitaxel are known to destroy particular compounds of the cytoskeleton [8–10,37]. Depending on the remaining structures of the cytoskeleton, the cells lose their ability to migrate. This affects the ability of the cells to migrate and to invade in a dose-dependent manner. After exclusion of direct cytotyxicity, we found a dose-dependent inhibition of the invasion rate with both drugs, vincristin and paclitaxel, in our assay. These findings show that the BSC allows to analyze drug-dependent effects on invasion rates quantitatively and is well suitable for functional invasion assays.

Conclusions

The BSC provides reproducible, cheap and rapid means to investigate the invasive behavior of glioma cells *in vitro*. Particular advantages of the BSC are the use of porcine brain which has a gray/white matter composition similar to human brain and the preserved three-dimensional and structural conditions of white matter tissue during invasion. The BSC is an *in vitro* model still differing from the clinical situation (use of permanent cell lines, lack of interaction of tumor cells with living brain cells). However, the characteristics of this new model provide high quality of data gained by BSC assays which should be close to the validity of *in vivo* data, reducing the wide gap between *in vitro* and *in vivo* invasion assays. Therefore, we consider this system to be a valuable addition to the armamentarium of test systems existing so far.

References

1. McComb RD, Burger PC: Pathologic analysis of primary brain tumors. Neurol Clin 3(4): 711–728, 1985
2. Deryugina EI, Luo GX, Reisfeld RA, Bourdon MA, Strongin A: Tumor cell invasion through matrigel is regulated by activated matrix metalloproteinase-2. Anticancer Res 17(5A): 3201–3210, 1997
3. Paulus W, Tonn JC: Basement membrane invasion of glioma cells mediated by integrin receptors. J Neurosurg 80(3): 515–519, 1994
4. Hu S, Chao CC, Khanna KV, Gekker G, Peterson PK, Molitor TW: Cytokine and free radical production by porcine microglia. Clin Immunol Immunopathol 78(1): 93–96, 1996
5. Niswender KD, Blackman SM, Rohde L, Magnuson MA, Piston DW: Quantitative imaging of green fluorescent protein in cultured cells: comparison of microscopic techniques, use in fusion proteins and detection limits. J Microsc 180(Pt 2): 109–116, 1995
6. Carenini S, Maurer M, Werner A, Blazyca H, Toyka KV, Schmid CD, Raivich G, Martini R: The role of macrophages in demyelinating peripheral nervous system of mice heterozygously deficient in p0. J Cell Biol 152(2): 301–308, 2001
7. Kobsar I, Berghoff M, Samsam M, Wessig C, Maurer M, Toyka KV, Martini R: Preserved myelin integrity and reduced axonopathy in connexin32-deficient mice lacking the recombination activating gene-1. Brain 126(Pt 4): 804–813, 2003
8. Tonn JC, Haugland HK, Saraste J, Roosen K, Laerum OD: Differential effects of vincristine and phenytoin on the proliferation, migration, and invasion of human glioma cell lines. J Neurosurg 82(6): 1035–1043, 1995
9. Silbergeld DL, Chicoine MR, Madsen CL: In vitro assessment of Taxol for human glioblastoma: chemosensitivity and cellular locomotion. Anticancer Drugs 6(2): 270–276, 1995
10. Terzis AJ, Thorsen F, Heese O, Visted T, Bjerkvig R, Dahl O, Arnold H, Gundersen G: Proliferation, migration and invasion of human glioma cells exposed to paclitaxel (Taxol) in vitro. Br J Cancer 75(12): 1744–1752, 1997
11. Laws ERJ, Goldberg WJ, Bernstein JJ: Migration of human malignant astrocytoma cells in the mammalian brain: Scherer revisited. Int J Dev Neurosci 11(5): 691–697, 1993
12. Rao JS: Molecular mechanisms of glioma invasiveness: the role of proteases. Nat Rev Cancer 3(7): 489–501, 2003
13. Rosenblum ML: Factors influencing tumor cell traffic in the central nervous system. J Neuro-Oncol 17(1993): 175–177 (090-3019090-3019), 1992
14. Paulus W, Tonn JC: Interactions of glioma cells and extracellular matrix. J Neurooncol 24(1): 87–91, 1995

15. Giese A, Westphal M: Glioma invasion in the central nervous system. Neurosurgery 39(2): 235–250, 1996
16. Goldbrunner RH, Bernstein JJ, Tonn JC: Cell-extracellular matrix interaction in glioma invasion. Acta Neurochir (Wien) 141(3): 295–305, 1999
17. Tonn JC, Goldbrunner R: Mechanisms of glioma cell invasion. Acta Neurochir Suppl 88: 163–167, 2003
18. Bernstein JJ, Goldberg WJ, Laws ERJ: Migration of fresh human malignant astrocytoma cells into hydrated gel wafers in vitro. J Neurooncol 18(2): 151–161, 1994
19. Deryugina EI, Bourdon MA, Reisfeld RA, Strongin: Remodeling of collagen matrix by human tumor cells requires activation and cell surface association of matrix metalloproteinase-2. Cancer Res 58(16): 3743–3750, 1998
20. Bauman GS, Fisher BJ, McDonald W, Amberger VR, Moore E, Del Maestro RF: Effects of radiation on a three-dimensional model of malignant glioma invasion. Int J Dev Neurosci 17(5–6): 643–651, 1999
21. Deryugina EI, Bourdon MA, Reisfeld RA, Strongin A: Remodeling of collagen matrix by human tumor cells requires activation and cell surface association of matrix metalloproteinase-2. Cancer Res 58(16): 3743–3750, 1998
22. Saito K, Oku T, Ata N, Miyashiro H, Hattori M, Saiki I: A modified and convenient method for assessing tumor cell invasion and migration and its application to screening for inhibitors. Biol Pharm Bull 20(4): 345–348, 1997
23. Sieuwerts AM, Klijn JG, Foekens JA: Assessment of the invasive potential of human gynecological tumor cell lines with the in vitro Boyden chamber assay: influences of the ability of cells to migrate through the filter membrane. Clin Exp Metastasis 15(1): 53–62, 1997
24. Sasaki CY, Passaniti A: Identification of anti-invasive but noncytotoxic chemotherapeutic agents using the tetrazolium dye MTT to quantitate viable cells in Matrigel. Biotechniques 24(6): 1038–1043, 1998
25. Giese A, Laube B, Zapf S, Mangold U, Westphal M: Glioma cell adhesion and migration on human brain sections. Anticancer Res 18(4A): 2435–2447, 1998
26. Giese A, Kluwe L, Laube B, Meissner H, Berens ME, Westphal M: Migration of human glioma cells on myelin. Neurosurgery 38(4): 755–764, 1996
27. Stoppini L, Buchs PA, Muller D: A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37(2): 173–182, 1991
28. Dore CF, Richards CD: Proceedings: an improved chamber for maintaining mammalian brain tissue slices for electrical recording. J Physiol (Lond) 239(2): 83P–84P, 1974
29. Freund E: Myeloarchitecture of the mesencephalon in the domestic swine (Sus scrofa domestica). Anat Anz 134(5): 445–459, 1973
30. Freund E: Topography of various nuclear areas in the mesencephalon of Sus scrofa domestica. Verh Anat Ges 63: 649–654, 1969
31. Yoshida D, Watanabe K, Noha M, Takahashi H, Teramoto A, Sugisaki Y: Tracking cell invasion of human glioma cells and suppression by anti-matrix metalloproteinase agent in rodent brain-slice model. Brain Tumor Pathol 19(2): 69–76, 2002
32. Murakami M, Goto S, Yoshikawa M, Goto T, Hamasaki T, Rutka JT, Kuratsu JI, Ushio Y: The invasive features of glial and non-central nervous system tumor cells are different on organotypic brain slices from newborn rats. Int J Oncol 18(4): 721–727, 2001
33. Yoshida D, Watanabe K, Noha M, Takahashi H, Teramoto A, Sugisaki Y: Anti-invasive effect of an anti-matrix metalloproteinase agent in a murine brain slice model using the serial monitoring of green fluorescent protein-labeled glioma cells. Neurosurgery 52(1): 187–196, 2003
34. Cotchin E: Spontaneous tumours in young animals. Proc R Soc Med 68(10): 653–655, 1975
35. Ziemer EL, Kramme PM, Higgins RJ, Smith BP: Astrocytoma in a boar. J Am Vet Med Assoc 187(8): 837–838, 1985
36. Jung S, Kim HW, Lee JH, Kang SS, Rhu HH, Jeong YI, Yang SY, Chung HY, Bae CS, Choi C, Shin BA, Kim KK, Ahn KY: Brain tumor invasion model system using organotypic brain-slice culture as an alternative to in vivo model. J Cancer Res Clin Oncol 128(9): 469–476, 2002
37. Chang SM, Kuhn JG, Robins HI, Schold SC, Jr, Spence AM, Berger MS, Mehta M, Pollack IF, Rankin C, Prados MD: A Phase II study of paclitaxel in patients with recurrent malignant glioma using different doses depending upon the concomitant use of anticonvulsants: a North American Brain Tumor Consortium report. Cancer 91(2): 417–422, 2001

Address for offprints: PD Dr. Roland Goldbrunner, Department. of Neurosurgery, University of Munich, Marchioninistr. 15, 81377 Munich, Germany; Tel.: +49-89-7095-3553; Fax: +49-89-7095-6550; E-mail: roland.goldbrunner@med.uni-muenchen.de