Evaluation of Poly (Glycerol-Adipate) Nanoparticle Uptake in an *In Vitro* 3-D Brain Tumor Co-Culture Model

W. Meng,*,[1] P. Kallinteri,*,[2] D. A. Walker,† T. L. Parker,‡ and M. C. Garnett*,[3]

*School of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, United Kingdom; †School of Human Development, Queen's Medical Centre, Nottingham, NG7 2UH, United Kingdom; and ‡School of Biomedical Science, University of Nottingham, Nottingham, NG7 2RD, United Kingdom

Despite the inherent problems associated with *in vivo* animal models of tumor growth and metastases, many of the current *in vitro* brain tumor models also do not accurately mimic tumor-host brain interactions. Therefore, there is a need to develop such co-culture models to study tumor biology and, importantly, the efficacy of drug delivery systems targeting the brain. So far, few investigations of this nature have been published. In this paper we describe the development of a new model system and its application to drug delivery assessment. For our new model, a co-culture of DAOY cell brain tumor aggregates and organotypic brain slices was developed. Initially, the DAOY aggregates attached to cerebellum slices and invaded as a unit. Single cells in the periphery of the aggregate detached from the DAOY aggregates and gradually replaced normal brain cells. This invasive behavior of DAOY cells toward organotypic cerebellum slices shows a similar pattern to that seen *in vivo*. After validation of the co-culture model using transmission electron microscopy, nanoparticle (NP) uptake was then evaluated. Confocal micrographs illustrated that DAOY cells in this co-culture model took up most of the NPs, but few NPs were distributed into brain cells. This finding corresponded with results of NP uptake in DAOY and brain aggregates reported elsewhere. Exp Biol Med 232:1100–1108, 2007

Key words: biodegradable nanoparticles; medulloblastoma; organotypic culture

Introduction

Malignant brain tumors in children younger than age 15 years account for 23% of brain tumors (1). Brain tumor therapy is currently limited in terms of the number of drugs and drug delivery systems to which can it can be applied. Development of delivery systems is hampered by the availability of suitable models for studying such systems *in vitro*.

The interaction between brain tumors and host tissue is likely to be of significance in therapy, particularly in defining differences between tumor and normal tissues that could be effectively exploited for therapy. In setting up such models we should be aware of and ensure that models have appropriate possibilities for tissue interaction, of which invasion is a principal component. The most valuable models are thus likely to be models which have been well characterized in terms of invasion properties. Numerous laboratory *in vivo* and *in vitro* models have been developed for the study of brain tumor invasion mechanisms (2–4).

In vivo models of brain tumor invasion currently use xenografts of primary human tumor cells transplanted to host rodent brains or use chemically induced primary tumors in animals. There are several disadvantages to the use of these animal models, such as a long latent period between administration of carcinogen and overt signs of neural neoplasia, the relatively low incidence of intracranial neoplasms (1, 3), and the obvious need to use immunosuppressed rats in transplanted brian tumors. *In vitro* models have a number of advantages, including easy accessibility for manipulation and study, and they do not raise as many experimental and ethical issues as *in situ* brain tumor models in animals. Therefore, *in vitro* models have been widely developed. The following brain tumor invasion models are commonly used as *in vitro* models: (i) 3-D spherical fetal brain aggregates or organotypic slice invaded by single brain tumor cells (4); (ii) 3-D confrontational spheroid brain tumor cultures (3, 5, 6); and (iii) organotypic brain slice cultures used to host astrocytoma spheroids (7).

[1] Current address: School of Pharmacy, University of London, London WC1N 1AX, United Kingdom.

[2] Current address: Medway School of Pharmacy, Universities of Kent/Greenwich, Chatham Maritime, Kent ME4 4TB, United Kingdom.

[3] To whom correspondence should be addressed at Boots Building, University Park, Nottingham, Nottinghamshire NG7 2RD, United Kingdom. E-mail: martin.garnett@nottingham.ac.uk

Received December 19, 2006.
Accepted April 25, 2007.

DOI: 10.3181/0612-RM-301
1535-3702/07/2328-1100$15.00

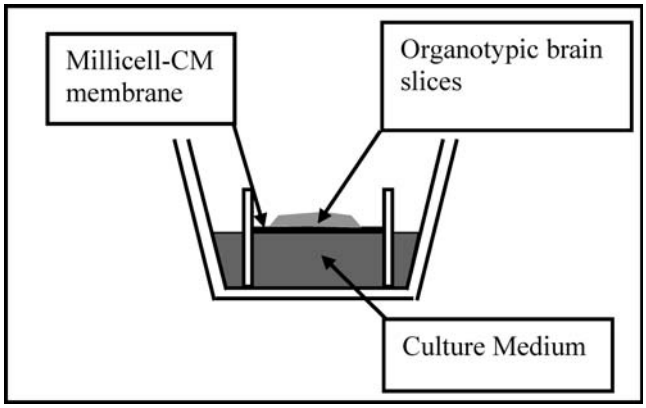

Figure 1. Technique used for culturing organotypic slices. Brain slices (400 μm), dissected from P2 Wistar rats were laid down on a small piece of porous and transparent Millicell-CM membrane, and the membrane was placed in the six-well plates. Medium was added to the bottom of the culture plate. Cultures were kept in a 5% CO_2 incubator at 37°C.

Here we report the use of medulloblastoma-derived cell (DAOY) spheroids co-cultured with rat organotypic cerebellum slice cultures

Uptake of rodamine B isothiocyanate (RBITC)-labeled nanoparticles (NPs) have been studied in DAOY cells and brain cells in monolayer cultures and 3-D cultures (8, 9). Those studies indicated that cell culture dimension played an important role in the uptake and penetration of NPs. Thus, the invasion model design is important, because cell culture microenvironment has an effect not only on the invasive behavior of brain tumor cells (10), but it may also have an effect on the NP uptake behavior of brain tumor cells and normal brain cells. These drug delivery issues have not been studied previously due both to the lack of suitable labeled particles and lack of an appropriate 3-D *in vitro* model.

An optimal model to study tumor cell invasion may be an organotypic model, since the interaction between tumor cells and host tissue and interaction between NPs and brain tumor cells/normal brain cells should be similar to the *in vivo* situation. In the present work we describe a co-culture model based on organotypic neonatal rat brain slices and 3-D spherical medulloblastoma cell line aggregates initially as a means of assessing the behavior of NPs for drug delivery. Medulloblastoma arises in the cerebellum and brainstem but not the cerebral cortex *in situ* (11, 12); therefore, organotypic cerebellum was chosen as the principal host tissue, but a comparison was made with cortical brain slices.

Materials and Methods

Materials. Poly(glycerol-adipate) (PGA), 12 kDa, 40% substituted with C_{18} acyl groups (13), was provided by by Dr. G. A. Hutcheon and Dr. S. Higgins (The Drug Delivery and Materials Science research group, Liverpool John Moores University, Liverpool, UK). P(S/6% DVB/V-COOH) Mag Microspheres (fluorescein isothiocyanate [FITC]-labeled magnetic microsphere) were obtained from Bangs Laboratories (Bangs Laboratories Inc., Fishers, IN). RBITC was purchased from Sigma-Aldrich (Poole, UK). DAPI (4′,6-diamidino-2-phenylindole, dilactate) was obtained from Molecular Probes (Paisley, UK). The DAOY cell line was purchased from American Type Culture Collection (Rockvillle, MD). All medium components and reagents for cell culture were obtained from Invitrogen Life Technologies Ltd. (Paisley, UK). All other chemicals were from Sigma-Aldrich (Poole, UK). All the reagents used for transmission electron microscopy (TEM) were from Agar Scientific Ltd. (Essex, UK).

Methods. *NP Preparation for Cellular Uptake.* NPs were prepared and characterized as described in our previous study (8). Briefly, NPs were formed by a nanoprecipitation method. Particle sizes were measured by photon correlation spectroscopy, and surface charges of NPs were determined by laser Doppler anemometry. For all experiments of NPs in the study of cellular uptake, PGA NPs were coated by incubation in 0.1% polysorbate-80 and passed through a syringe filter (0.2 μm; Vivascience, Hanover, Gemany) to sterilize the NP suspension.

Monolayer Cell Culture. DAOY cells, a human cerebellar medulloblastoma cell line, were maintained on minimum essential medium (MEM) supplemented with 15% fetal bovine serum (FBS), 200 m*M* L-glutamine, 0.1 m*M* nonessential amino acids, 1.0 m*M* sodium pyruvate, and 7.5% sodium bicarbonate solution at 37°C and 5% CO_2.

Aggregate Culture. DAOY cells were grown in monolayer culture and detached from the substratum, as described above. The individual cells (2 ml, 1×10^6 cells/ml) were cultured in DAOY culture medium in 25-ml screw-top culture flasks (Scientific Laboratory Supplies, Wilford, UK) and maintained at a constant rotation of 70 rev/min on an orbital shaker (Cole-Palmer, Vernon Hills, IL) at 37°C. Cultures were observed and medium was exchanged after 24 hrs of culture.

Organotypic Slice Culture. Organotypic brain slices were prepared from 2-day-old Wistar rats. After the brain was rapidly removed and immersed in ice-cold Hanks' balanced salt solution (HBSS), including 10 m*M* HEPES, cerebellum and cerebral cortex were cut into 400-μm–thick slices with a tissue chopper (McIlwain Tissue Chopper; Mickle Laboratory Engineering Co. Ltd., Surrey, UK) under sterile conditions in a laminar flow hood. Three slices then were laid down on a Millicell-CM membrane insert (Millipore, Carrigtwohill, Ireland), and the insert was placed in individual wells of six-well plates (Fig. 1). Medium (1 ml) was added to the bottom of culture plate (14). Slices were cultured in the same culture medium as mixed brain cells at 37°C in a 5% CO_2 incubator. The slices were cultured for 10 days to allow for macrophage removal of cell debris. Use of animals for tissue samples was carried out in accordance with appropriate UK legislation, guidelines, and Home Office license.

A Co-Culture Model of Tumor Aggregates and Organotypic Brain Slice. To accurately evaluate drug delivery systems *in vitro*, we have developed a co-culture model of tumor aggregates on organotypic brain slice. On Day 1, cerebellum slices were dissected from P2 rat brains and cultured as described above. On Day 3, DAOY monolayer cells were seeded as subconfluent in a 25-cm^2 tissue culture flask. Culture medium was replaced with fresh culture medium (5 ml), including FITC-labeled magnetic microspheres (40 μl, 1% w/v) on the following day. After 24 hrs of incubation of DAOY monolayer cells with magnetic microspheres, DAOY cells were harvested and cultured in 25-ml screw-top culture flasks for 24 hrs to form DAOY aggregates. On Day 6, DAOY aggregates (2 μl) ranging in size from 200 to 300 μm were gently added on the surface of each slice. The co-culture model of DAOY aggregates and organotypic brain slices was cultured for another 2, 4, or 6 days. The culture technique is shown in Figure 2.

Uptake of RBITC-Labeled NPs by Co-Culture Model. When DAOY aggregates were added onto the surface of organotypic brain slices, they were cultured for 4 days. On the third day, NP suspension (200 μg) was added on the top of the co-culture model and incubated for 24 hrs. The co-culture model was then washed three times with phosphate-buffered saline (PBS), fixed in 1% freshly prepared paraformaldehyde (PFA) on the following day after incubation with NPs, and treated for microscopy investigation.

Morphologic Studies. Aggregates and co-culture models were incubated with DAPI (300 n*M*) for 30 mins after samples were fixed. Samples were subsequently rinsed with PBS three times and visualized and imaged under a Leica SP2 MP confocal microscope (Leica Microsystems, Milton Keynes, UK) using 488-nm filter (fluorescein), 543-nm filter (RBITC), and UV laser (DAPI).

TEM. After DAOY aggregates were cultured on the organotypic cerebellum or cortex slices for 2, 4, or 6 days, they were rinsed with PBS three times. The slices were fixed in 2% PFA and 3% glutaraldehyde in 0.1 *M* cacodylate buffer for 1 hr and then were postfixed in 1% osmium tetroxide for 1 hr. The slices were dehydrated in a graded series of ethanol solutions (60%, 80%, and 100%). They were then soaked in a mixture of 100% 1,2-epoxypropane and agar-embedding resin at a 1:1 ratio overnight at 4°C, after which they were soaked three times in fresh neat agar resin for 2 hrs. The slices were placed in embedding wells with fresh neat agar resin, and wells were placed in a vacuum oven at 60°C for at least 48 hrs for polymerization of the resin. The polymerized blocks were cut into thin sections (80–100 nm thick). Then sections were placed on Formvar-coated copper grids (Agar Scientific Ltd., Stansted, UK), stained with an aqueous solution of 2% uranyl acetate for 15 mins, washed briefly in water, stained with Reynolds' lead citrate for 5 mins, and finally washed in water before visualization. Samples were imaged under TEM (JEOL JEM1010, Jeol UK Ltd., Welwyn, UK) with magnifications ranging from ×20,000 to ×300,000.

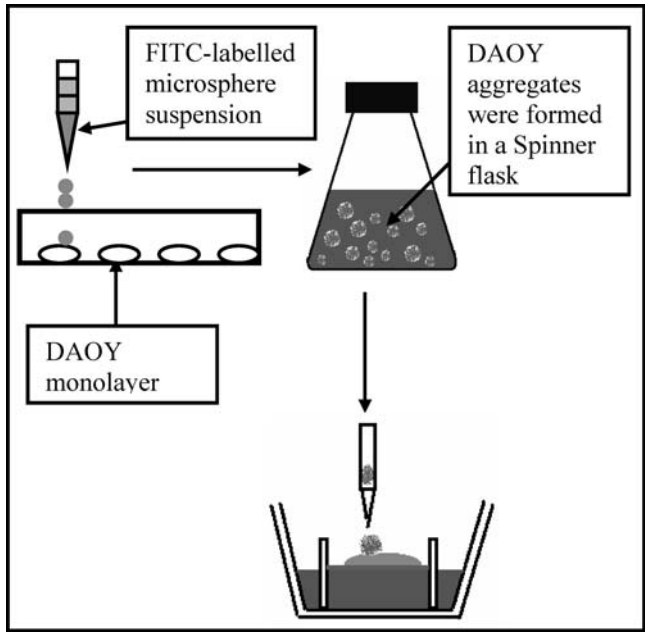

Figure 2. Techniques used for culturing co-culture model of DAOY aggregates and organotypic brain slices. After DAOY monolayer cells were incubated with FITC-labeled microspheres (40 μl, 1%) for 24 hrs, cells were harvested and cultured in a Spinner flask for 24 hrs to form aggregates. DAOY aggregate suspension (2 μl) was gently added on the surface of brain slices on the following day. Then the co-culture models were maintained in 5% CO_2 incubator at 37°C for 2, 4, or 6 days.

Results

Characteristics of DAOY Aggregates and Organotypic Cerebellar Slices. FITC-labeled magnetic microspheres were added to DAOY cells in monolayer culture and were incubated with cells for 24 hrs. DAOY aggregates were then formed from the above-mentioned DAOY cells in monolayer cultures by a rotation method. As shown in Figure 3a, DAOY aggregates were well formed, individual cells still could be seen in the periphery of the aggregate, and cells in the center of aggregates were darker under phase-contrast microscopy. TEM micrographs showed that FITC-labeled magnetic microspheres were taken up by DAOY cells and sorted into lysosome compartments (Fig. 3b), thus enabling easy recognition of labeled cells in TEM sections.

After 48-hr culture, the viability of organotypic cerebellum slices was assessed using a light microscope. As shown in Figure 3c, cell outgrowth was evident as neurites extending from the edge of the cerebral cortex slices after 48-hr culture. A TEM image (Fig. 3d) also confirmed the culture viability. Slices appeared densely packed, with cells of varying size and morphology. Large mononuclear cells had a large, dense nucleus and cytoplasm filled with organelles. Cellular and organelle integrity appeared to have been maintained even after 14 days in

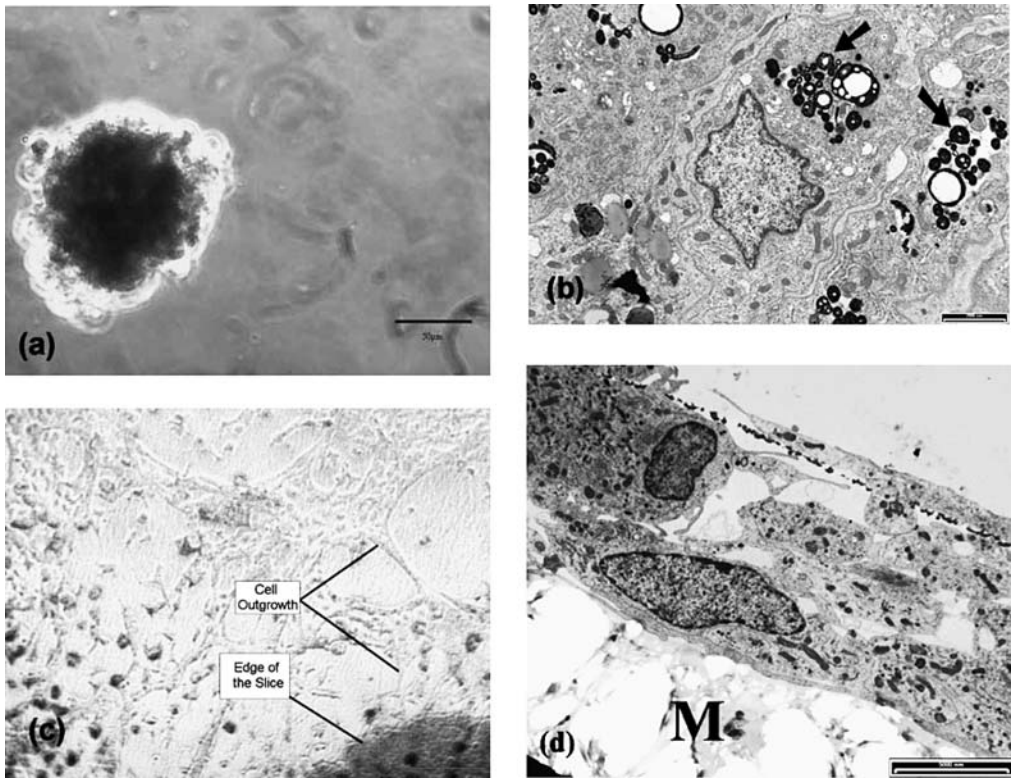

Figure 3. Characteristics of aggregates and organotypic slice culture. (a) A phase-contrast micrograph showing the DAOY aggregates. Scale bar = 50 μm. (b) TEM micrograph of DAOY aggregates containing magnetic microspheres. Arrows indicate the FITC-labeled magnetic microspheres that were taken up by DAOY cells and sorted into lysosomes. Scale bar = 2000 nm. (c) A phase-contrast micrograph illustrating culture viability of organotypic slices. Scale bar = 50 μm. (d) TEM micrograph of organotypic slices. Scale bar = 5000 nm. M, membrane insert.

culture. All these data indicate that the method used in our work was successful in maintaining and growing cerebellum slices from 2-day neonatal rats.

Co-Culture Model of DAOY Aggregates and Organotypic Cerebellum Slices. *Evaluation of Co-Culture Model Using Fluorescence Microscopy.* Fluorescence microscopy images illustrated trends in the invasive behaviour of DAOY aggregates over time *in vitro* with cerebellum slices (Fig. 4). Individual DAOY cells invaded cerebellum slices over time by varying amounts and distances. (i) Individual DAOY cells started to invade cerebellum slices over 2 days. Only one single DAOY cell showing green fluorescence in the image was found in host tissue, and it had invaded over a relatively short distance (210 μm) from the DAOY aggregate margin (Openlab 3.1.7 package; Improvision, UK). (ii) Invasion of individual DAOY cells into slices appeared more clearly after 4 days of co-culture time, with a longer invasion distance. Most individual DAOY cells reached 109 to 136 μm, and a few DAOY cells were up to 427 μm away from the DAOY aggregate margin after 4 days of co-culture. (iii) After 6 days of incubation, DAOY cells had massively invaded the cerebellum slices. The invasion distance of most cells was within 455 μm, but some of the DAOY cells reached 972 μm away from the aggregate margin.

Evaluation of Co-Culture Model Using TEM. A second way of ascertaining the invasive properties of the co-culture model was visualizing the co-culture model under TEM. Figure 6 demonstrates the process of DAOY aggregates invading into cerebellum slices. To compare DAOY aggregate invasion over time, three areas were commonly chosen (Fig. 5): (i) whole image of co-culture model with lower magnification; (ii) area right under DAOY aggregate; and (iii) area close to the brain-tumor border.

After 2 days of co-culture, a firm attachment of DAOY aggregates to the cerebellum slice was observed. The cerebellum slice kept much of its organotypic structure at the border between DAOY aggregate and cerebellum slice. A few single DAOY cells infiltrated the cerebellum slice (Fig. 6 Ia and Ib), but no DAOY single cells were observed in the area that was close to the brain-tumor border (Fig. 6 Ic). With longer co-culture time, DAOY aggregates invaded the cerebellum slice, and normal brain slices were destroyed at the brain-tumor border. In addition to many DAOY single cells invading the cerebellum slices, some DAOY cells were also discerned at the bottom of the cerebellum slice (Fig. 6 IIa, IIb, IIIa and IIIb). DAOY single cells could also be seen in the area close to the brain-tumor border (Fig. 6 IIc and IIIc).

Microscopic Investigation of RBITC-Labeled NPs in Tumor Aggregates and Organotypic Cerebral Cortex Slices Co-Culture Model. To investigate DAOY aggre-

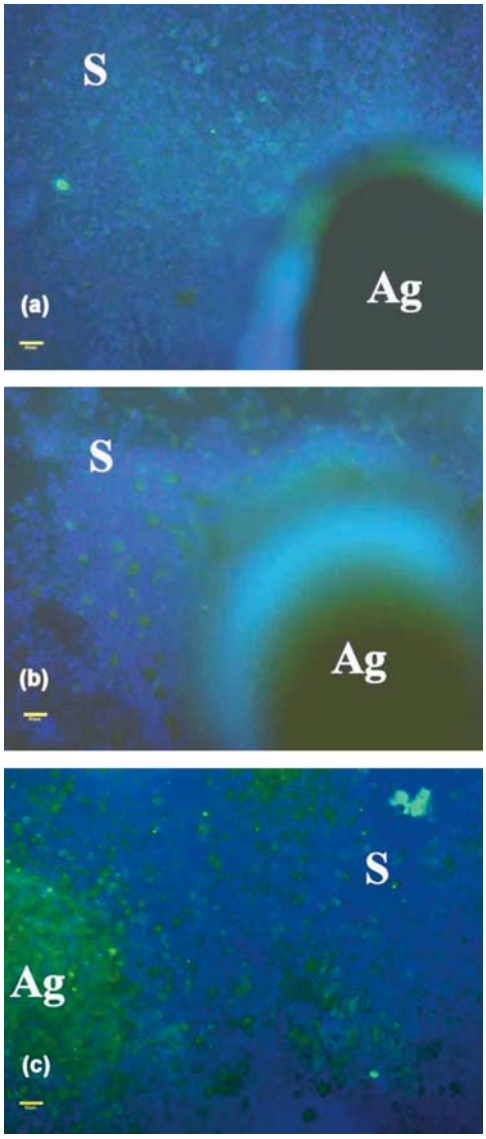

Figure 4. Fluorescence micrographs showing co-culture of DAOY aggregates (Ag) and organotypic cerebellum slices (S). (a) 2 days. (b) 4 days. (c) 6 days. Scale bar = 100 μm. Green or dark green fluorescence was from FITC-labeled magnetic microspheres; blue fluorescence was from DAPI.

gate invasive behavior in organotypic cerebral cortex slices, co-culture models of DAOY aggregates and cerebral cortex were cultured under the same conditions as the cerebellum slice co-culture model. TEM micrographs of the co-culture model were taken from the same areas as described in the cerebellum slice co-culture model. DAOY aggregates attached to the cerebral cortex slices after a 2-day co-culture (Fig. 7Ia). With an increase of co-culture time, DAOY aggregates invaded into cerebral cortex slices (Fig. 7IIa through IIIa). Cortex slices under DAOY aggregate were destroyed by DAOY cells, and the majority of normal brain cells were gradually replaced by the DAOY aggregate. Even after a 6-day co-culture time, no DAOY individual cells

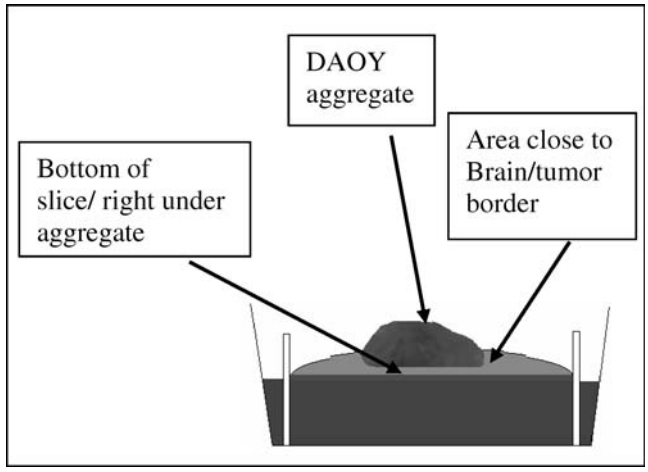

Figure 5. Cartoon demonstrating the areas of TEM image taken from the co-culture model.

could be found in the area close to the brain-tumor border (Fig. 7IIb through IIIb).

Uptake of RBITC-Labeled NPs by Co-Culture Model. Fluorescence microscopy and TEM studies ascertained that DAOY cells invaded into cerebellum slices, and massive invasion occurred after a 6-day co-culture time. To clearly understand the different response of DAOY cells and normal brain cells to RBITC-labeled NPs, a 4-day co-culture model was chosen to carry out the NP uptake study, because at this time DAOY aggregate invasion into cerebellum slice occurred, but the invasion pattern was not as extensive as that in 6-day co-culture model. RBITC-labeled NP suspension was added on the surface of the co-culture model on the third day of culture time, and then the co-culture model was incubated with RBITC-labeled NPs for 24 hrs. The co-culture model was fixed and visualized under confocal fluorescence microscopy on the fourth culture day. Figure 8 shows the uptake of NPs by the co-culture model. Since DAOY cells were labeled with FITC-labeled magnetic microspheres and normal brain cells were not, cells with green and blue (DAPI) fluorescence represented DAOY aggregates or DAOY single cells, and brain cells had only blue fluorescence. It shows that DAOY cells on the edge of aggregates took most of the RBITC-labeled NPs (red fluorescence), and few NPs were taken up by DAOY cells in the middle of aggregate and normal brain cells (Fig. 8).

Discussion

To our knowledge, suitable *in vitro* models to investigate the behavior of drug delivery using NPs in contact with both normal and tumor tissue simultaneously have not previously been reported. In considering this work, we wanted a model that had relevance to *in vivo* tumor biology and was accessible to set up for routine studies. Extensive experience in our laboratories with organotypic brain slices suggested that this would be a good model for

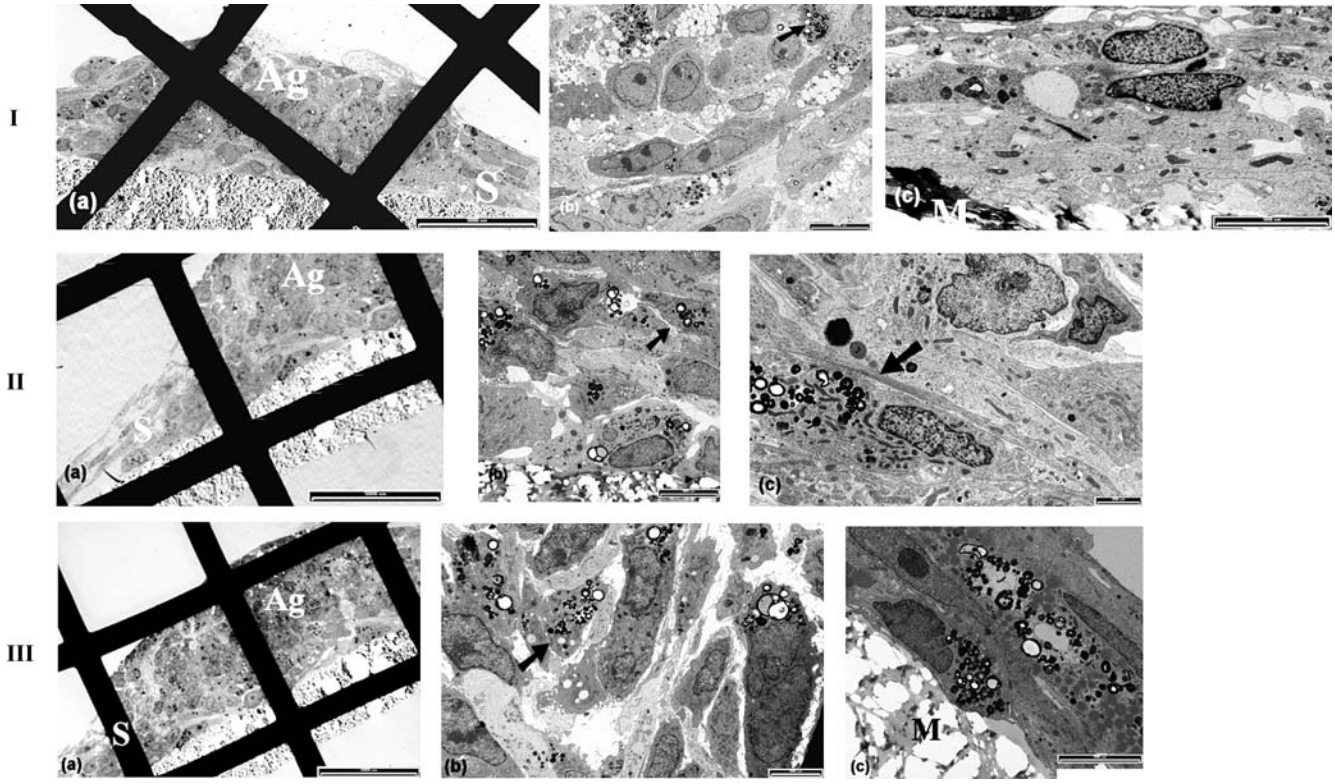

Figure 6. TEM micrographs of DAOY aggregate (Ag) and cerebellum slice (S) co-cultured for (I) 2 days; (II) 4 days; and (III) 6 days. (a) Co-culture model with lower magnification. Scale bar: 50,000 nm. (b) Area of slice right under the DAOY aggregate with high magnification. Scale bar: 10,000 nm. (c) Area of slice close to the brain-tumor border with high magnification. Scale bar = 5000 nm. It was shown that DAOY cells (arrow) invaded into slices. M, membrane insert

normal brain tissue. Human brain tissue slices would be the best choice to match with human tumor cell lines or aggregates, but normal human brain tissue is not routinely available. Both young and adult brain slices do not proliferate and survive well in culture, so neonatal rat brain slices were selected. A similar pattern of immunoreactivity for glial cells and neurofilament markers for neurones was seen in 3-D neonatal spheroid cultures for three different brain regions: cerebellum, cortex, and brainstem, as seen in adult rat tissue (low glia fibrillary acid protein [GFAP], high neurofilament expression). However, cells grown in 2-D monolayer showed the opposite pattern (high GFAP, low neurofilament expression; Ref. 15)

A previously reported model (7) has used 1.0-mm brain slices, but sections of this thickness become necrotic and lose neuronal function relatively rapidly. In contrast, the 0.4-mm slices selected for this study can be cultured for months without necrosis or loss of viability and represent a true organotypic system (16).

The labeling of tumor cells was another important consideration. The work described by Jung *et al.* (7) used cells labeled by transfection with green fluorescent protein; however, we chose to used FITC-labeled iron microparticles so that we could monitor cell invasion by both confocal fluorescence microscopy and TEM within the same experiment. This also meant that the cancer cells used in the study were not further genetically transformed.

Our primary therapeutic interest is in medulloblastoma, so we chose DAOY cells as a well-characterized cultured cell line. There are some doubts about the lineage and background of this line, but is both metastatic and conforms to the genetic fingerprint of native medulloblastoma cells (17).

Assessment of Tumor Spherical Aggregates and Organotypic Brain Slice Co-Culture Models. Invasion is defined as the destruction of host cell extracellular matrix (ECM) and active translocation of tumor cells through the ECM. It is not simply due to expansion of tumor by growth or by a passive distribution of tumor cells (18). Medulloblastoma arises in the cerebellum and brainstem but not the cerebral cortex *in situ* (19). This growth pattern, together with previous work by us with different invasion models (15) led to the expectation that DAOY aggregates would invade organotypic cerebellum slices but may not invade cortex slices so readily.

Fluorescence microscopy and TEM studies not only confirmed that DAOY cells invade into organotypic cerebellum slices but also showed the invasion behavior of DAOY aggregates (Figs. 4 and 6). DAOY aggregates as a unit adhered to adjacent cerebellum slices after 2 days of co-culture and gradually destroyed and invaded into normal

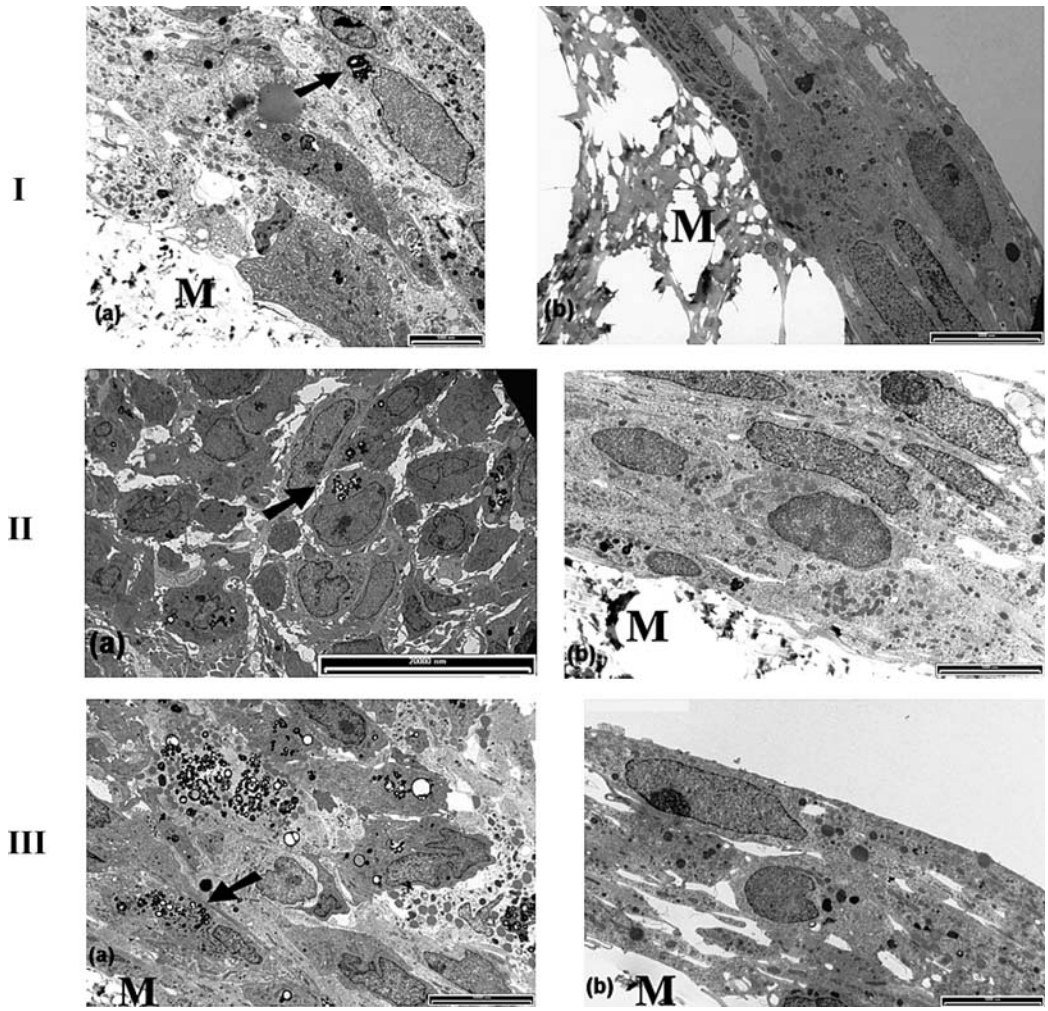

Figure 7. TEM micrographs of DAOY aggregate and cerebral cortex co-cultured for (I) 2 days; (II) 4 days; and (III) 6 days. (a) Area of slice right under the DAOY aggregate with high magnification. Scale bar = 10,000 nm. (b) Area of slice close to the brain-tumor border with high magnification. Scale bar = 5000 nm. It was shown that DAOY cells (purple arrow) invaded into the bottom of slices, but no DAOY cells were observed in the area close to the brain-tumor border even after 6 days of co-culture. M = membrane insert.

cerebellum slice from the tumor-brain border. Individual DAOY cells spread widely and invaded almost the whole cerebellum slices over a relatively long distance after 6 days of co-culture (Fig. 4). Terzis *et al*. (6) reported a similar invasion pattern of DAOY aggregates after 7 days of co-culture of DAOY aggregates and normal whole-brain aggregates.

Invasion of tumor cells into normal tissue is a complicated process, and invasion follows a three-step model (1, 20, 21): tumor cells (i) detach from the growing primary tumor mass; (ii) adhere to the ECM *via* specific receptors; and (iii) locally degrade ECM of adjacent cells and migrate into adjacent host tissue. The results reported in the present organotypic co-culture tumor invasion model have the features defined above associated with a true invasion by cancer cells and are similar to the invasive behavior of medulloblastoma *in vivo* (22).

TEM studies also showed that DAOY aggregate invasion into cerebral cortex slices occurred, but DAOY cell invasion was relatively limited compared with the co-culture model of DAOY aggregates and cerebellum slices (Fig. 7). Tumor invasion is a complicated process, governed by molecular cross-talk between DAOY cells and DAOY cells, DAOY aggregates and normal brain cells, and DAOY cells and normal brain cells; by types and amount of lytic enzymes produced by DAOY aggregates and DAOY cells; and by fine regulation of adhesion to and detachment from ECM (23). Histologic differences between cerebellum slices and cerebral cortex slices result in biologic differences. Therefore, the microenvironment created by cerebellar and DAOY aggregates differed from the corresponding microenvironment created by cerebral cortex slices and DAOY aggregates, resulting in the different invasion patterns observed.

TEM results of DAOY cells invading cerebral cortex slices were different from those in a previous report, in which it was found that DAOY aggregates did not attach to brain cortex aggregates (15). As mentioned in previous

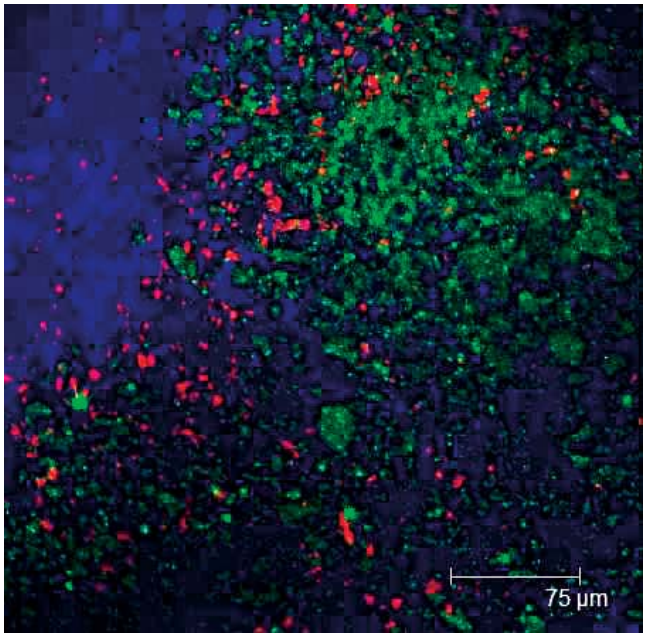

Figure 8. Confocal micrograph demonstrating RBITC-labeled NPs taken up by a 4-day co-culture model of DAOY aggregates and organotypic cerebellum slices. Cells containing green (from FITC-labeled magnetic microspheres) and blue (from DAPI) fluorescence represent DAOY cells. Normal brain cells had only blue fluorescence. Red fluorescence is from RBITC-labeled NPs. It was shown that DAOY cells take up most NPs, and few NPs were taken up by normal brain cells.

discussion, cell culture dimension plays a critical role in cytoarchitecture of regional brain cultures, in turn affecting types of brain cell, ratio between each brain cell, composition of ECM, and subsequent invasive behavior of DAOY cells. Several co-culture models have been used to investigate the role of dimension on medulloblastoma invasion, including: (i) co-culture of DAOY single cells and brain monolayer cells from cerebellum or cerebral cortex; (ii) co-culture of DAOY single cells and spherical aggregates dissociated from different parts of the brain; and (iii) co-culture of DAOY aggregates and regional brain aggregates. Parker *et al.* reported that invasion occurred in the first two culture models but not in the third one (15). Very little is known about the mechanisms involved in medulloblastoma cell invasion so far; therefore, the discrepancy in results between ours and Parker's could be attributed to the difference in culture model resulting in different culture histology and microenvironment created by tumor cells and regional brain cells (24).

Microscopic Investigation of RBITC-Labeled NPs in Tumor Aggregate and Organotypic Cerebellum Slice Co-Culture Model. Other studies by us using aggregates have demonstrated that the number of NPs taken up by DAOY aggregates was five times higher than that taken up by mixed whole fetal brain aggregates after 24 hrs of incubation time, whereas in 2-D culture the foetal brain cells took up more NPs than the DAOY cells (9). The majority of the NPs were taken up by the invading DAOY cells at the periphery of the aggregate, where it had become integrated with the normal brain slice, with many fewer particles associated with the brain slice. The few particles seen associated with the middle of DAOY aggregates compared with the massive uptake of NPs by DAOY cells in the periphery of DAOY aggregates could possibly be attributed to the "fried egg" pattern of the co-culture model seen in Figure 6 Ia, IIa, and IIIa. With increased co-culture incubation time, DAOY cells in the periphery of the aggregate invaded into the cerebellum slice, whereas DAOY aggregates in the middle still kept a humped shape raised above the tissue slice. Thus, at the periphery the cells were at the same level as the tissue slice cells, but the center of the aggregate the cells were at a higher elevation. The liquid interface is at the bottom of the slide, so once NP suspension was added from the top of the co-culture model it would then drain to the liquid level. The brain slice and DAOY cells in the periphery of aggregates, therefore, had more chance and longer incubation time with NP suspension than DAOY cells in the middle of aggregate. Despite this artefact, it is seen that this model does still show the high NP uptake ratio of brain tumor cells to brain cells. Thus, it is suggested that culture models of 3-D spheroids and organotypic slices would be a useful tool to determine the specificity of NP uptake by target cells *in vitro*.

Relevance to Drug Delivery. There is now a substantial body of evidence that untargeted drug delivery systems in the NP size range, such as liposomes, can accumulate in tumor tissues both in experimental animals and patients (25) due to differences in vascularity and vascular (dis)organization (26). However, there is little information in the literature on how materials of NP size behave in tissues in terms of uptake into tumor cells once they have accumulated in the tumor. It has been suggested in the older literature that uptake of macromolecules is very high in tumor cells (Ref. 27 and references therein) and that this differential rate of endocytosis could be exploited therapeutically.

Other *in vitro* experiments by us show that there is a differential uptake both between tumor and normal tissue but only in 3-D culture, and these also suggest a difference between different normal cell types (9). The results of the present NP uptake study were in concordance and showed that in a co-culture model more representative of tumor growth *in vivo* there is a still a selective uptake advantage, even for untargeted NPs. The present work, therefore, suggests that because PGA NPs were preferentially distributed in the brain tumor tissue, this could lead to better therapeutic benefit of NP delivery systems by localizing the drug in the vicinity of the cancer cells and reducing side effects.

Invasion Studies—Future Work. The preliminary characterization of this model using TEM suggests that this may also be further developed as a useful model for invasion studies. However, further work still needs to be carried out regarding characterization of the tissues and details of the invasion process. The work on conversion of

this model to use human brain tissue slices as host and use of primary human tumor material to make it a more representative model of human cancer invasion is underway.

Conclusion. The aim of this study was to develop an *in vitro* 3-D brain tumor invasion model suitable for evaluation of PGA NPs as a drug delivery system. In a study of the co-culture model of DAOY aggregates and cerebellum slices, fluorescence microscopy and TEM studies demonstrated DAOY aggregates attached and invaded as a unit into slices. In addition, DAOY single cells in the periphery of aggregates detached from DAOY aggregates and gradually replaced normal brain cells over time. After 6 days of co-culture, the invasive distance from the DAOY aggregate margin of some DAOY single cells could reach to 972 μm. The invasion behavior of DAOY cells to organotypic cerebellum has a similar pattern to that reported *in vivo*. This, therefore, suggested that the co-culture model of DAOY spherical aggregates and organotypic cerebellum slice can be used as a representative model to study the behavior of NPs.

The next stage was to begin to investigate the uptake of NPs in this co-culture model.

Confocal micrographs demonstrated that tumor cells had higher rates of NP uptake than normal host cells, which corresponds to results of NP uptake in DAOY and mixed brain aggregates reported elsewhere (9).

All of the results presented in this study suggest that this novel 3-D *in vitro* co-culture model could be used to effectively evaluate the selectivity of a drug delivery system between tumor cells and brain cells, which can provide a greater chance to evaluate anticancer therapeutic potential of a drug delivery system *in vitro*. The model could also be further characterized and developed as a model of tumor invasion.

The poly(glycerol-adipate) polymers were generated through a BBSRC-funded collaborative project in collaboration between the University of Nottingham and Liverpool John Moores University. We would like to thank Dr. S. Higgins and Dr. G. Hutcheon for the synthesis and provision of these polymers. We also thank the Children's Brain Tumour Research Trust in Nottingham for funding for consumables and instrumentation of the NP uptake and release studies.

1. Pilkington GJ. Tumour cell migration in the central nervous system. Brain Pathol 4:157–166, 1994.
2. Engebraaten O, Bjerkvig R, Lund-Johansen M, Wester K, Pedersen PH, Mork S, Backlund EO, Laerum OD. Interaction between human brain tumour biopsies and fetal rat brain tissue in vitro. Acta Neuropathol (Berl) 81:130–140, 1990.
3. Pilkington GJ, Bjerkvig R, de Ridder L, Kaaijk P. In vivo and in vitro models for the study of brain tumour invasion. Anticancer Res 17: 4107–4110, 1997.
4. Jung S, Kim HW, Lee JH, Kand SS, Rhu HH, Jeong JI, Yang SY, Chung HY, Bae CS, Choi C, Shin BA, Kim KK, Ahn KY. Brain tumour invasion model system using organotypic brain-slice culture as an alternative to in vivo model. J Neurosurg 94:80–89, 2001.
5. Penar PL, Khoshyomn S, Bhushan A, Tritton TR. Inhibition of glioma invasion of fetal brain aggregates. In Vivo 12:75–84, 1998.
6. Terzis AJA, Arnold H, Laerum OD, Bjerkvig R. Interaction between human medulloblastomas and foetal rat brain aggregates in vitro. Acta Neurochir (Wien) 126:11–16, 1994.
7. Jung S, Ackereley C, Ivanchuk S, Mondal S, Becker LE, Rutka JT. Tracking the invasiveness of human astrocytoma cells by using green fluorescent protein in an organotypical brain slice model. J Cancer Res Clin Oncol, 128:469–476, 2002.
8. Meng W, Parker TL, Kallinteri P, Walker DA, Higgins S, Hutcheon GA, Garnett MC. Uptake and metabolism of novel biodegradable poly (glycerol-adipate) nanoparticles in DAOY monolayer. J Contr Rel 116: 314–321, 2006.
9. Meng W, Kallinteri P, Walker D, Garnett MC, Parker TL. A new in vitro 3-D brain tumour co-culture model used to study nanoparticle uptake and tumour invasion. Neuro Oncol 8:293–372, 2006.
10. Sutherland RM. Cell and environment interactions in tumour microregions: the multicell spheroid model. Science 240:177–184, 1988.
11. Chang CH, Housepian EM, Herbert C Jr. An operative staging system and a megavoltage radiotherapeutic technique for cerebellar medulloblastoma. Radiology 93:1351–1359, 1969.
12. Nystrom B, Olson L, Ungerstedt U. Noradrenaline nerve terminals in human cerebral cortices: first histochemical evidence. Science 172: 924–926, 1972.
13. Kallinteri P, Higgins S, Hutcheon GA, St Pourcain CB, Garnett MC. Novel functionalized biodegradable polymers for nanoparticle drug delivery systems. Biomacromolecules 6:1885–1894, 2005.
14. Stoppini L, Buchs PA, Muller D. A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37:173–182, 1991.
15. Parker TL, Searle KH, Storer LCD, Walker DA. Regional brain spheroid cultures: an in vitro 3D model to study specificity of tumour invasion. Neuro Oncol 7:306–307 2005.
16. Gahwiler BH, Capogna M, Debanne D, Mckinney R, Thompson SM. Organotypic slice cultures: a technique has come of age. Trends Neurosci 20:471–477, 1997.
17. MacDonald TJ, Brown KM, LaFleur B, Peterson K, Lawlor C, Chen Y, Packer RJ, Cogen P, Stephan, DA. Expression profiling of medulloblastoma: PDGFRA and the RAS/MAPK pathway as therapeutic targets for metastatic disease. Nat Genet 29:143–152, 2001.
18. Kaczarek E, Zapf S, Bouterfa H, Tonn J, Westphal M, Giese A. Dissecting glioma invasion: interrelation of adhesion, migration and intercellular contacts determine the invasive phenotype. Int J Dev Neurosci 17:625–641, 1999.
19. Kleihues P, Cavenee WK. World Health Organisation Classification of Tumours: Pathology and Genetics of Tumours of the Nervous System Lyon: IARC Press, 2000.
20. MacDonald TJ, Declerck YA, Laug WE. Urokinase induce receptor mediated brain tumor cell migration and invasion. J Neurooncol 40: 215–226, 1998.
21. Bellail AC, Hunter SB, Brat CT, VanMeir EG. Microregional extracellular matrix heterogeneity in brain modulates glioma cell invasion. Int J Biochem Cell Biol 36:1046–1069, 2004.
22. Bolteus AJ, Berens ME, Pilkington G.J. Migration and invasion in brain neoplasms. Curr Neurol Neurosci Rep 1:225–232, 2001.
23. Mareel MM, Van Roy FM, Bracke ME. How and when do tumor cells metastasize? Crit Rev Oncog 4:559–594, 1993.
24. Fidler IJ. Cancer metastasis. Br Med Bull 47:157–177, 1991.
25. Harrington, KJ, Syrigos KN, Vile RG.. Liposomally targeted cytotoxic drugs for the treatment of cancer. J Pharm Pharmacol 54:1573–1600, 2002.
26. Baban DF, Seymour LW. Control of tumour vascular permeability. Adv Drug Del Rev 34:1513–1518, 1998.
27. Trouet A, Deprez-de Campeneere D, de Duve C. Chemotherapy through lysosomes with a DNA-daunorubicin complex. Nat New Biol 239:110–112, 1972.