2. Materials and Methods

2.1. Materials

2.1.1. Reagents and Chemicals

Product name	Name used in the text	Supplier
Acetic acid	Acetic acid	Roth, Germany
Acrylamide/Bisacrylamide 30% solution	Acrylamide	Sigma, Germany
Agar agar	Agar	Roth, Germany
Agarose	Agarose	Roth, Germany
Ammoniumpersufat	APS	Merck
ATX Ponceau S red staining solution	Ponceau	Fluka, Germany
Bacto-Tryptone	Bacto-Tryptone	BD biosciences
Bacto-Yeast	Bacto-Yeast	BD biosciences
Beta mercaptoethanol	Mercaptoethanol	Sigma, Germany
Brij 35	Brij 35	Sigma, Germany
Clodronic acid disodium salt	Clodronate	Roche Diagnostics, Germany
Brilliant blue R	Coomassie blue	Sigma, Germany
Cholesterol	Cholesterol	Sigma, Germany
Cyanoacrylate glue	Super glue	Henkel, Germany
ECL Plus Western Blotting Detection Reagents	ECL reagent	Amersham Biosciences, USA
Ethylenglycol	Ethylenglycol	Roth, Germany
Gel blotting paper	Blotting paper	Roth, Germany
Gelatin from porcine skin, Type A	Gelatin	Sigma, Germany
Glycerol	Glycerol	Sigma, Germany
Glycine	Glycine	Merck
Hybond-P PVDF membrane	PVDF membrane	Amersham Biosciences, USA

Product name	Name used in the text	Supplier
Hyperfilm ECL	ECL film	Amersham Biosciences, USA
Lipofectamine 2000 transfection reagent	Lipofectamine	Invitrogen, USA
Methanol	Methanol	Roth, Germany
Natriumchlorid	Sodium chloride	Roth, Germany
Paraformaldehyde	PFA	Merck
Phosphatidylcholine	Phosphatidylcholine	Lipoid EPC; LIPOID, Germany
Rainbow molecular weight marker	Molecular weight marker	Amersham Biosciences, USA
Saran wrap	Transparent foil	Dow Chemical Co, USA
Sodium dodecyl sulfate	SDS	Sigma, Germany
Sucrose	Sucrose	Merck
N,N,N',N'-Tetramethylethylenediamine	TEMED	Amresco, USA
TRIS-aminomethane	TRIS	Roth, Germany
Triton X-100	Tx-100	Merck
Tween 20	Tween 20	Merck

2.1.2. Kits

Product name	Name used in the text	Supplier
BCA Protein Assay Kit	Protein concentration assay	Pierce, USA
Endo Free Plasmid Maxi kit	Maxiprep kit	Qiagen, Germany
Live/Dead Viability/Cytotoxicity kit	Live-dead kit	Molecular Probes, USA

Product name	Name used in the text	Supplier
Matrix Metalloproteinase-14, Biotrak Activity Assay System	MMP14 activity assay	Amersham, USA
QIAprep Spin Miniprep kit	Miniprep kit	Qiagen, Germany
RNeasy Mini kit	RNA purification columns	Qiagen, Germany

2.1.3. Primary antibodies

Antigen	Host	Dilution	Supplier
Glial acidic fibrillary protein (GFAP)	Rabbit	1:500	Dako Cytomation, Denmark
Green fluorescent protein (GFP)	Goat	1:1000	Acris Antibodies, Hiddenhausen, Germany
Ionized calcium binding adaptor molecule one (Iba-1)	Rabbit	1:500	Waco, Japan
MT1-MMP Hinge region, Human and mouse	Rabbit	1:1000	Sigma, Germany
Neurofilament 200kDa-NF 200	Rabbit	1:200	Chemicon, Temecula, CA, USA
Von Willebrand factor-(vWf)	Rabbit	1:200	Chemicon, Temecula, CA, USA

Antigen	Host	Conjugate	Dilution	Supplier
Rabbit IgG	Donkey	Rhodamine red	1:125	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
Rabbit IgG	Donkey	FITC	1:125	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
Mouse IgG	Mouse	Horse radish peroxidase-HRP	1:10000	Sigma, Germany
Rabbit IgG	Donkey	Biotin	1:2000	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
Substances	Conjugate		Dilution	Supplier
Iso lectin B4	Biotin		1:50	Sigma, Germany
Streptavidin	HRP		1:10000	Amersham biosciences, USA
Streptavidin	Rhodamine Red		1:200	Jackson ImmunoResearch Laboratories, West Grove, PA, USA

2.1.4. Equipment

Product name	Name used in the text	Supplier
Biophotometer	Biophotometer	Eppendorf, Germany
Mini protean 3 Cell	Electrophoresis chamber	Biorad, USA
Trans blot SD	Electrophoresis transfer electrodes	Biorad, USA
Power Pack 300	Power Pack	Biorad, USA
AGFA Curix 60	Film developing machine	AGFA, Japan

Product name	Name used in the text	Supplier
RC-5B Superspeed Centrifuge	Ultracentrifuge	Sorvall, Germany
Centrifuge 5417R	Centrifuge	Eppendorf, Germany
Leica SM 2000R	Sliding microtome	Leica Co., Germany
Leica VT1000S	Vibratome	Leica Co., Germany
Stereotactic head holder	Stereotactic head holder	David Kopf Instruments, USA
Jung CM3000	Cryostat	Leica, Germany
Sarastro 2000	Confocal microscope	Molecular Dynamics, USA
TCS SP2	Confocal microscope	Leica, Germany
Axioplan	Fluorescence microscope	Zeiss, Germany
Axiovert 100	Inverted fluorescence microscope	Zeiss, Germany
Two-Photon microscope	Two-Photon microscope	Till Photonics GmbH, Germany
Wallac Victor 1420 Multilabel counter	Microplate reader	Perkin Wallac GmbH, Freiburg, Germany

2.1.5. Cell culture equipment

Product name	Name used in the text	Producer
Air incubator	incubator	Heraeus, Hanau, Germany
Disposable Filter Unit, 0,2µm and 12µm cellulose acetate	Sterile filters	Scheicher&Schnell, Germany
Ethibond Excel 2-0	sewing cone	Johnson&Johnson International
Falcon cell culture inserts, 0,4µm, 6 well format	(6 well) inserts	BD labware
Falcon Multiwell-6well and 24 well	6 and 24 well plate	BD labware

Product name	Name used in the text	Supplier
Mikroliterspritze 7001N	1µl syringe	Hamilton, Switzerland
Tissue culture dishes, 60 and 30mm	Petri dishes	TPP, Switzerland
Tissue culture flasks 25cm^2 and 75cm^2	T 25 and T 75	TPP, Switzerland
Tissue Tek	Tissue Tek	Sakura, The Netherlands
Aqua Poly/Mount	Mounting medium	Polysciences, Inc, USA

2.1.6. Cell culture media

Product name	Name used in the text	Supplier
DNase	DNase	Worthington, USA
Dulbecco´s minimum essential medium, with 4500mg/L Glucose and L-Glutamine	DMEM	Gibco, Gaithersburg, MD
Dulbecco´s Phosphate Buffered Saline, without $CaCl_2$ and $MgCl_2$	PBS	Gibco, Gaithersburg, MD
Ethylenediaminetetraacetic acid	EDTA	Sigma, Germany
Fetal bovine serum	FCS	Gibco, Gaithersburg, MD
Genetecine, G-418 Sulphate	G418	Gibco, Gaithersburg, MD
Hank´s balanced salt solution, without $CaCl_2$ and $MgCl_2$	HBSS	Gibco, Gaithersburg, MD
L-Glutamine	Glutamine	Serva, Germany
Opti MEM 1	Opti MEM	Gibco, Gaithersburg, MD
Insulin	Insulin	Gibco, Gaithersburg, MD
Penicillin, Streptomycin, Glutamine-100X	P/S/G	Gibco, Gaithersburg, MD
Trypsin	Trypsin	Roche

2.1.7. Buffers and Media

2.1.7.1. Bacteria propagation

SOC medium: 2g Bacto-Tryptone, 0,5g Bacto-Yeast extract, 1ml 1M NaCl, 0,25ml 1M KCl, add in 97ml ddH_2O, autoclave and cool to RT. Add 2M Mg^{2+} and 2M glucose stocks. Filter the complete medium through 0,2µm filter and adjust to pH 7,0.

Luria-Bertani (LB) medium for 1L: 10g Bacto-Tryptone, 5g Bacto-Yeast extract, 5g NaCl adjust pH to 7,5 with NaOH. Autoclave to sterilize, cool to 55^o C and add antibiotics suitable for the expression plasmid.

2.1.7.2. Cell culture media

Medium-1: DMEM supplemented with 10% heat inactivated FCS, 0,2mM glutamine, 100U/ml penicillin, and 100 µg/ml streptomycin

Medium-2: DMEM containing 25% heat inactivated horse serum; 50mM sodiumbicarbonate; 2% glutamine; 25 % HBSS; 1µg/ml Insulin; 2,46mg/ml glucose; 0,8µg/ml Vitamin C; 100units/ml penicillin; 100µg/ml streptomycin and 5mM Tris.

2.1.7.3. Immunolabeling

TBS+: 100mM Tris, 150mM NaCl (pH 7,4), 0,1%Tx100,

TBS++: 100mM Tris, 150mM NaCl (pH 7,4), 0,1%Tx-100, pH 7,4 and 3% donkey serum

TBST: 100mM Tris, 150mM NaCl, pH 7,4, 0,05% Tween 20

Western Blotting blocking buffer: 5% Fat free milk powder in TBST

2.1.7.4. Gelatine zymography

Sample buffer: 2,5ml 0,5M TRIS-HCl (pH 6,8); 2,0ml Glycerol; 4,0ml 10%SDS; 100µl 10%Tx-100; 100µl 1%Brom Phenol Blue add H_2O to 10ml

5X Running buffer: 125mM TRIS HCl (pH 8,3), 1,23M Glycine, 0,5% SDS for 1L:15,1g TRIS, 94g Glycine, 5g SDS

Developing buffer: 50mM TRIS HCl (pH 7,6), 10mM $CaCl_2X2H_2O$,50mM NaCl, 0,05% Brij 35 for 1L: 6,06g TRIS, 1,47g $CaCl_2$, 2,92g NaCl, 0,5g Brij 35 (pH=7,6)

0,5M TRIS (pH 6,8)-for lL: 60,55g Tris in 800ml H20 adjust pH to 6,8 with HCl than add H_20 to 1L

1,5M TRIS (pH 8,8)-for 1L:181,65g Tris in 800ml H20 adjust pH to 8,8 with HCl than add H_20 to 1L

Gelatin stock solution: vortex 20mg gelatine with 1ml H_2O in an 2ml eppendorf cap, and shortly boil in the microwave

Washing buffer: 2,5% Tx-100 in H_2O; dilute 25ml of Tx-100 into 975ml H_2O

Coomasie staining solution: Dilute 0,5g of Coomassie blue stain in 40% methanol, 10% acetic acid solution

Destaining solution: 40% Methanol and 10% acetic acid solution

2.1.7.5. Western Blotting

Sample buffer: 1% SDS, 1% Tx-100, Complete proteinase inhibitor (Roche) in TBS (pH 7,4)

10X Running buffer: 30g TRIS, 140g glycine, 10g SDS add to 1l H_2O

Transfer buffer: 2,93g glycine, 5,81 TRIS, 0,375g SDS add 800ml H_2O and 200ml methanol

Lower gel buffer: 72,7g TRIS, 1,6g SDS add 400ml H_20 (pH 8,8)

Upper gel buffer: 18,2g TRIS, 1,2g SDS add 300ml H_2O (pH 6,8)

10% APS: 100mg APS in 1ml H_2O

2.1.8. Software

Product name	Name used in the text	Supplier
Olympus Fluo View 300 Tiempo 4.0	Olympus Fluo View 300 Tiempo 4.0	Olympus, Japan
Adobe Photoshop	Photoshop	Adobe Systems Inc. USA
Image Pro plus	Image Pro plus	Media Cybernetics, USA
Microsoft Excel	Excel	Microsoft, USA
SPSS	SPSS	SPPS Inc., USA

2.2. Methods

2.2.1. Microglial cell culture

Microglial cultures were prepared from cerebral cortices of newborn C57/Bl6 mice as described previously (Prinz and Hanisch 1999). Mice were decapitated and skin and skull were removed simultaneously. Then, the brain was dissected from the brain stem and transferred to cold HBSS. The cerebellum and olfactory bulb were taken off. Then, the blood vessels and meninges were carefully removed from the hemispheres. The tissue was collected in a 15ml flask and washed 3 times with HBSS. After the final wash 200-300µm Trypsin/DNAse and 200µm DNAse was applied, and left at RT for 4 min. The reaction was blocked with 2ml of medium-1. The tissue was mixed carefully 10 times with a plastic Pasteur pipette and then 5 times with a fire-polished glass Pasteur pipette. Medium was added to fill the 15ml Falcon tube and centrifuged (10 min, 800rpm, 4°C). The supernatant was discarded, the pellet resuspened with 2ml medium and plated in T 75 culture flasks (2-3 mouse brain preparations per flask). Cultures were incubated at 37°C, 5% CO_2 and the next day adherent cells were washed 5 times with PBS. After culturing for one week, 10ml medium-1 and 5ml of L929 (fibroblast cell line) conditioned medium was applied for two days. The microglia are now seen as floating cells or as semi-adherent cells on top of an astrocytic monolayer. Microglia are harvested by shaking the culture flask at 37°C, 100 rpm for 30 min and centrifuging the medium at 800 rpm, 4°C for 10min. The cells were seeded in 6-well plates at a density 10^6 cells/well. Cultures usually contain > 95 % microglial cells, which can be stained with Iso lectin B4, a marker for microglia. For experiments cultures were used 1 to 5 days after plating.

2.2.2. Glioma cell lines

The F98 cell line was obtained from American Type Culture Collection (Manassas, VA), the GL261 cell line was purchased from National Cancer Institute, NCI-Frederick (MD, USA). F98 and GL261 glioma cells were grown in medium-1 in T 25 tissue culture flasks. The rat F98 cell line was selected for its high basal motility rate in vitro, the mouse GL261 cell line for its isogenity to the donor animals (C57/BL6 mice) of the brain slices. The medium was changed every two days and cells were passaged, when the cell density in the flask reached confluency. Cell cultures were

maintained in the incubator at 37°C in humidified and by 5% CO_2 conditioned atmosphere.

2.2.3. Amplification of vectors

The p-EGFP-N1 vector contains the human cytomegalovirus promoter, which drives high-level expression of the enhanced green fluorescent protein (EGFP) in transfected cells (Fig. 2.1.). Chemically competent bacteria (DH-5α Chemically Competent E.Coli, Invitrogen) transformed with the vector were selectively propagated by a vector mediated resistance for kanamycin/neomycin. In short, 100µl chemically competent bacteria were transformed with 100ng of pEGFP-N1 plasmid DNA. Cells were heat shocked for 1min in a 42°C water bath and immediately returned on ice for 2min. Then, 1ml LB medium was added and bacteria were incubated for 45min at 37°C (rotatory shaker, >200rpm). The mixture was centrifuged 3min at 7000rpm and excess medium was removed. The bacteria were then plated on selective LB plates at 1:1, 1:10 and 1:100 dilutions and overnight (ON) incubated at 37°C. Single colonies were picked and propagated in selective SOC medium for 12h on a shaker at 37°C. Finally, plasmid DNA was isolated using Mini-prep kit (Qiagen) and the yield was measured in a Biophotometer.

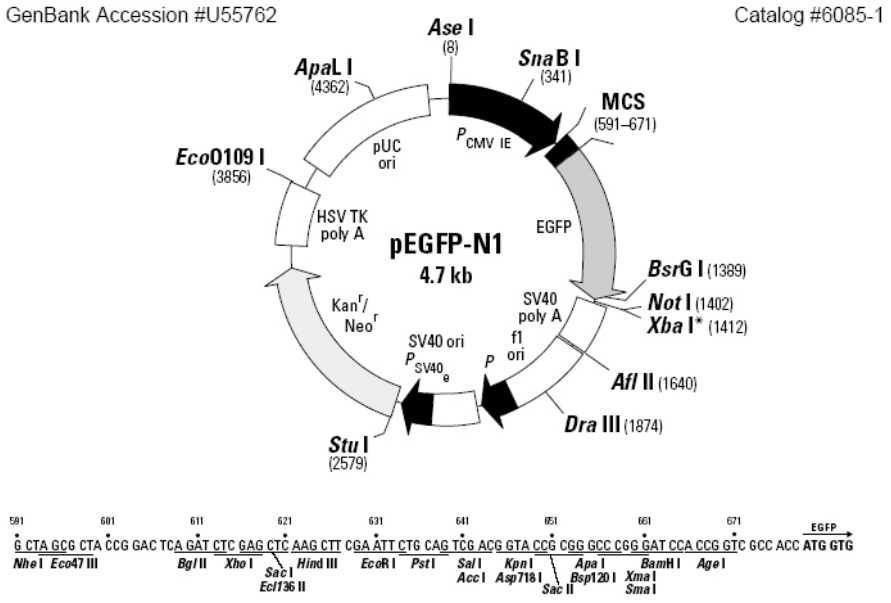


Fig. 2.1. Restriction map and Multiple Cloning Site (MCS) of pEGFP-N1 vector

2.2.4. G418 sensitivity test

Prior to the transfection, F98 and GL261 cells were challenged with increasing concentrations of G418, from 0,2 – 2,0mg/ml. The minimal concentration of G418 which was required to kill all the cells after two weeks in culture was taken as a

sorting concentration for the selection of stable transfectants. In the case of F98 it was 1,2mg/ml and 600µg/ml for GL261. Briefly, F98 and GL261 cells were trypsinized, centrifuged, counted and plated in 24- well plate at the concentration of $2X10^4$cells/well. Then, they were exposed to increasing concentrations of G418 (see table 2.2.). The medium was changed twice a week. After two weeks in culture, the minimal concentration of G418 enough to kill all the cells was established. That concentration was later used as selecting concentration of G418 for culturing transfected F98 and GL261.

Control- no G418	Control- no G418	0,2mg/ml G418	0,2mg/ml G418	0,4mg/ml G418	0,4mg/ml G418
0,6mg/ml G418	0,6mg/ml G418	0,8mg/ml G418	0,8mg/ml G418	1,0mg/ml G418	1,0mg/ml G418
1,2mg/ml G418	1,2mg/ml G418	1,4mg/ml G418	1,4mg/ml G418	1,6mg/ml G418	1,6mg/ml G418
1,8mg/ml G418	1,8mg/ml G418	2,0mg/ml G418	2,0mg/ml G418	2,2mg/ml G418	2,2mg/ml G418

Table2.2. Distribution of increasing concentrations of G418 in a 24- well plate

2.2.5. Transfection of glioma cell lines

On the day before transfection 10^6 cells were plated in a 6-well plate so that they reached 90-95% confluency before the transfection. Different concentrations (see table 2.1.) of Lipofectamine 2000 were mixed with Opti-MEM I medium in separate vials and incubated 5 min at room temperature. Meanwhile 20µg of plasmid DNA were mixed with 1250µl of Opti-MEM I. Then, 250µl of diluted DNA was added to each vial containing Lipofectamine 2000 and incubated for 30min. In the meantime, the normal growing medium of the plated cells was exchanged for 2ml/well of Opti-MEM I. The transfection mixture of Lipofectamine 2000 and plasmid DNA (500µl per group) was applied dropwise onto the cells with simultaneous gentle shaking of the 6-well plate (Table 2.1.). The cells were exposed to the Lipofectamine/plasmid DNA mixture for 6h, then the reagents were exchanged for normal growth medium. After overnight incubation, cells were trypsinised, pelleted and plated in T 25 flasks,

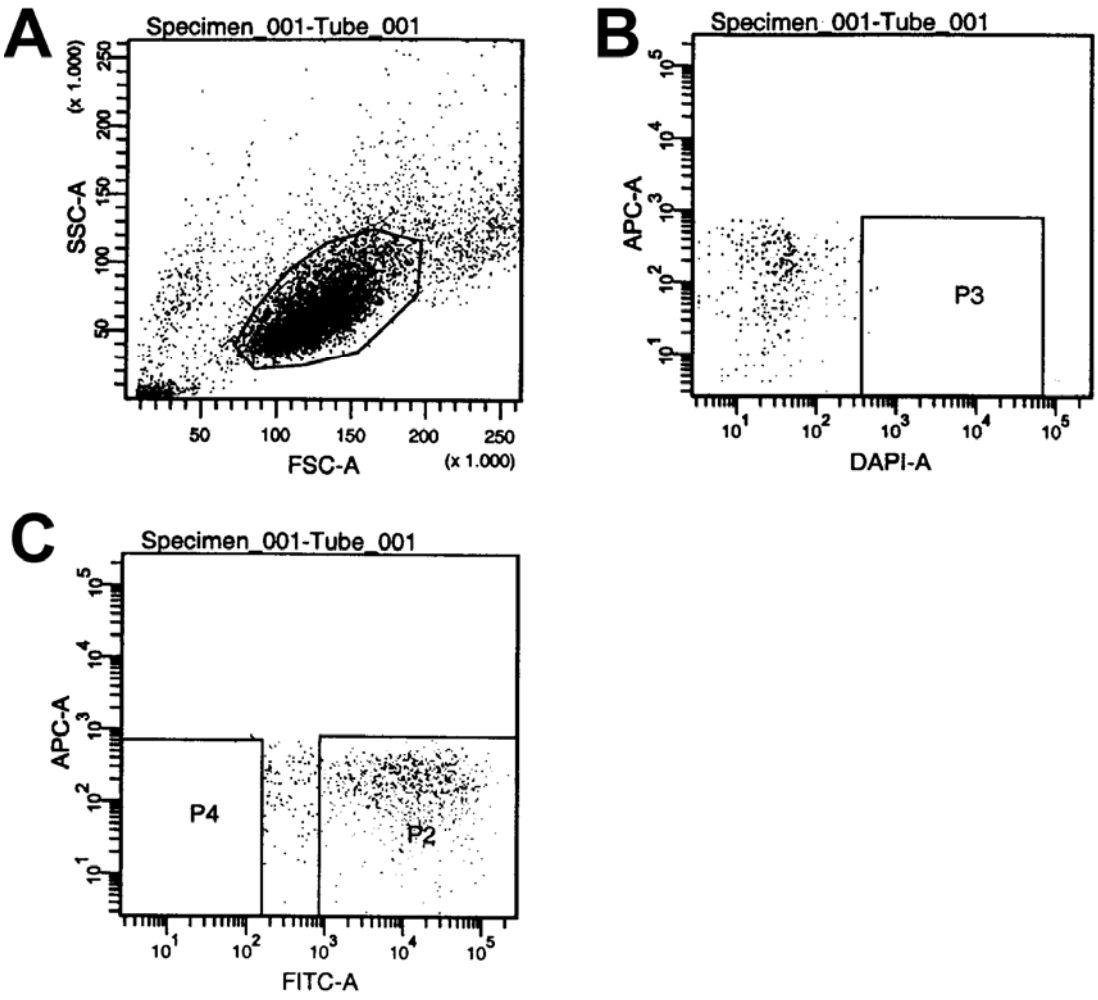
with the growth medium containing the appropriate concentration of G418 (DMEM, 10%FCS, 3mg/L L-Glutamine, 1,2mg/ml for F98 and 0,6mg/ml for GL261 of G418). During the next three weeks, the cells were selected for their ability to express the pEGFP-N1 vector. When the number of cells expressing EGFP reached about 50%, the cells were FACS sorted.

vial	DNA	Opti-MEM I	Lipofectamine 2000	Opti-MEM I
#1	4µg	250µl	2µl	250µl
#2	4µg	250µl	4µl	250µl
#3	4µg	250µl	8µl	250µl
#4	4µg	250µl	12µl	250µl
#5	4µg	250µl	16µl	250µl
Control	0	250µl	8µl	250µl

Table2.1. Concentration gradient of Lipofectamine 2000 for a transfection in a 6-well plate

2.2.6. Fluorescence Assisted Cell Sorting (FACS) of EGFP expressing glioma cells

To prepare glioma cells for FACS, they were trypsinised, centrifuged (10min, 4°C, 800rpm) and the pellet was carefuly but thoroughly resuspended in 5ml PBS containing 0,05% EDTA. To eliminate cell clumps, the cell suspension was filtered through a cell strainer with a 40µm mash. Then, the single-cell suspension of glioma cells was subjected to FACS (Fig. 2.2.). The EGFP fluorescent cells were collected and further cultured in medium containing DMEM, 10%FCS and G418. Due to decreased EGFP expression in later passages (after 10^{th} passage), the FACS sorted glioma cells were only used until 5^{th} passage.



D

Tube: Tube_001

Population	#Events	%Parent	%Total
All Events	10.000		100,0
P1	7.149	71,5	71,5
P2	1.909	26,7	19,1
P3	20	0,3	0,2
P4	4.965	69,8	49,6
P1 AND P2	1.924	19,2	19,2
P1 AND P4	4.965	49,6	49,6

Fig. 2.2. FACS of the EGFP expressing glioma cells. (A) The glioma cells with similar shape and size (P1) were chosen for further fluorescence sorting. (B) The FACS device recognizes only few cells (P3) when the cells are plotted to (non applied) DAPI label- negative control. (C) 26,7% (beginning) are well-shaped and green fluorescent (26,7% of chosen 71,5% cells). (D) Detailed statistic distribution depicts that 19,2% were collected with the FACS (19,2 % of the total cell amount).

2.2.7. The organotypical brain slice model

The procedure to establish organotypical brain slice cultures is a modification of previously published protocols (Stoppini, Buchs et al. 1991; Jung, Ackerley et al. 2001; Murakami, Goto et al. 2001; Jung, Kim et al. 2002; Yoshida, Watanabe et al. 2003). Brain tissue was derived from 16-day-old male C57BL/6 mice (animal breeding facility, Schönwalde and Charles River, Germany) and from transgenic mice expressing EGFP under a promoter for glial fibrillary acidic protein (EGFP-GFAP mice; (Nolte, Matyash et al. 2001). For the organotypical brain slice cultures preparations these mice were decapitated and the skin on the head was cut and removed. Then, the skull was opened and the brain was taken out of the brain cavity using a spatula and placed in ice cold PBS. The cerebellum was cut off with a razor blade. The cerebrum was glued with super glue onto a cutting block on its caudal side and supported from the back side by a 2% agar block. Now, coronal sections of 250µm were cut with a vibratome. The brain slices were collected with a glass pipette (with its wider opening) and transferred onto a 0,4µm polycarbonate membrane in the upper chamber of a transwell tissue insert, which was inserted into a 6-well plate. Excess PBS was removed in a laminar flow bench. The brain slices were cultivated at the air-liquid interface in 1ml of medium-1 per well. After overnight equilibration of the brain slices in medium-1, it was exchanged for cultivation medium (medium- 2).

2.2.7.1. Liposomes

Clodronate loaded liposomes were obtained from GOT Therapeutics (Berlin, Germany) and from the Deptartment of Molecular Cell Biology of the Free University of Amsterdam. For the preparation of clodronate liposomes 86mg of phosphatidylcholine and 8mg of cholesterol were combined with 10ml of a clodronate solution (0,7M; a gift from Roche Diagnostics, Mannheim, Germany) and gently sonicated. The resulting liposomes were then washed to eliminate free drug. For control experiments PBS filled liposomes were prepared under the same conditions. All liposomes were passed through a 12µm filter immediately prior to use in order to eliminate large lipid aggregates (Van Rooijen and Sanders 1994).

2.2.7.2. Glioma cell injection into brain slices

T 25 culture flasks confluent with EGFP expressing glioma cells were trypsinised, centrifuged and the pellet was resuspended into 200µl medium. The cell number was adjusted to 10^4/µl. 5000 F98 or GL261 glioma cells within a defined injection volume (0,5µl) were inoculated into the slices using a 1µl syringe with a blunt tip mounted to a self constructed micromanipulator. To ensure identical experimental conditions, the gliomas were always inoculated into the same anatomical area. Therefore the tip of the syringe was always placed at the same defined region on the slice surface. The EGFP expressing glioma cells were inoculated below the corpus callosum into the globus pallidus. Using the micromanipulator an injection canal was formed, reaching 150µm deep into the 250µm thick slice. Then, the needle was retracted by 50µm leaving an injection cavity of approximately 50µm. The cell suspension was slowly injected over 30s and subsequently the syringe was slowly pulled out in 10µm incremental steps over 60s. Directly after injection the glioma cells remained at the inoculation site, which could therefore mark the point of origin for all further movements of these cells (Fig. 3.6. A-E). Careful control of the injection procedure ensured that no cells spilled onto the surface of the slices, which could then migrate over this surface rather than invade through the tissue.

2.2.8. In vivo Inoculation of GL261 cells into C57/BL6 mouse brain

2.2.8.1. Anesthesia

C57/BL6 mice (P16-20) were anestheteized with i.p. injections of 0,1% Xylazyne and 1,5% Ketamine- hydrochloride mixture in 0,9%NaCl. 10µl of the anesthetic mixture was injected per 1g of mouse body weight. The eyes of the mice were carefully covered with glycerin fat to avoid cornea drying.

2.2.8.2. Glioma inoculation into the mouse brain

Anesthetised C57/BL6 mice were immobilized and mounted onto a stereotactic head holder in the flat-skull position. The skin of the skull was dissected with a scalpel blade and the skull surface was disinfected with 10% KI solution. 1 mm anterior and 1.5 mm lateral to the bregma, the skull was carefully drilled with 20G needle tip. Then a 1-µl syringe with a blunt tip was inserted to a depth of 4 mm and retracted to a

depth of 3 mm from the dural surface. During 2min 1µl ($2x10^4$ cells/µl) of GL261 EGFP cell suspension was slowly injected. The needle was then slowly taken out from the injection canal and the skin was sutured with a surgical sewing cone.

2.2.8.3. Paraformaldehyde fixation

The mice were euthanised by 10% Ketamine i.p. injection and perfused with an intra-cardiac injection of 0,9% NaCl solution. Then, the NaCl perfusate was replaced by freshly prepared 4% PFA and each mouse was perfused with 30ml of 4% PFA. Now, the brain was carefully removed and postfixed for an overnight period in 4% PFA. Finally, the brains were cryopreserved in 30% sucrose solution (48h incubation minimum).

2.2.8.4. Immunohistochemistry of brain sections (Floating sections)

The PFA perfused cryoprotected brains were rapidly frozen in dry ice and mounted onto a sliding microtome. 40µm thick sections were collected into CPC-cryoprotecting solution (25% Glycerol and 25% Ethylenglycol in 0,05M phosphate buffer). Before immunolabeling, the sections were first three times washed in TBS+ and subsequently incubated in TBS++ (blocking buffer) for 1h. Then, the sections were incubated 48-72h at 4°C with primary antibodies. Sections were washed three times in TBS+ and incubated overnight with fluorescently conjugated secondary antibodies. Now, they were washed three times in TBS and finally, mounted onto microscope glass slides, covered with coverslips and stored at 4°C until used for microscopical analysis.

2.2.9. Immunofluorescence of organotypical brain slice cultures

2.2.9.1. Tissue preparation for immunolabeling

Organotypical brain slice cultures were fixed for 3h in 4%PFA and then washed 3x15min in TBS+ buffer. Following fixation, the organotypical brain slice cultures were directly immunolabelled (see bellow) or they were cryoprotected by overnight incubation in 30% sucrose solution. After cryoprotection, the slices were embedded in Tissue Tek and rapidly frozen in dry ice. Using a cryostat device, the preparations

were cut into 16µm thick sections and mounted onto microscope slides. Before the immunolabeling, the sections were postfixed for 15 min on the microscope slides to prevent detachment of the section from the slide.

2.2.9.2. Immunolabeling

All stainings were performed on whole brain slice cultures or on cryosectioned organotypical brain slice cultures. The primary antibodies were applied at the indicated dilutions (see Table 2.4.5. Antibodies) overnight in TBS ++ buffer (4°C). This was followed by three washing cycles in TBS+. Then, the appropriate fluorescent conjugated secondary antibody was applied (1:125) for 3h at RT. Finally, the slides were then washed three times in TBS and mounted with mounting medium.

2.2.10. Microscopy

2.2.10.1. Two- Photon microscopy

A Two-Photon microscope was used to study the distribution of glioma cells at the inoculation site. EGFP was exited at the two photon specific wavelength peak level of around 850nm. Reconstruction of the three dimensional distribution was achieved by using the Olympus Fluo View 300 Tiempo 4.0 software.

2.2.10.2. Confocal microscopy

Migratory distances were investigated using a confocal microscope (Sarastro 2000, USA). The EGFP fluorescence in glioma cells was excited with a wavelength of 488nm and detected at 530nm. Immunohistochemical preparations were visualized using a confocal microscope with three different laser channels for FITC (485nm/535nm), TRIC (555nm/575nm) and Cy5 (650 nm /665 nm; TCS SP2; Leica).

2.2.10.3. Fluorescence microscopy

Immunohisto/cytochemical preparations were also visualized with a fluorescence microscope. Fluorescence microscopy was further used to evaluate transfection rates, which were measured by a corresponding EGFP expression in the glioma cultures. Live cultures were analysed for fluorescence using an inverted fluorescence microscope (Axiovert 100).

2.2.11. Quantification of glioma cells invasion in organotypical brain slice cultures

Confocal microscopy images obtained at 10 time optical magnification were stored as Tagged Image Files (tif) with a resolution of 72 pixels / inch. Since one tumor was usually to large to be photographed with only one picture, series of micrographs were made, covering the complete tumor. Using Photoshop, the images were merged into one picture which represented the whole tumor. Finalized images were analyzed with the Image Pro plus software. This software allowed us, after calibration, to measure the migratory distances of single glioma cells to the injection (starting) point. Then, the values, expressed in μm, relating to migratory distances of single cells were collected and exported into MS Excel. Cells were grouped according to their migratory distances in 100μm incremental steps. Migration-Histograms were constructed from these raw data.

2.2.12. Gelatin zymography

Activity of gelatinases (MMP-2 and MMP-9) was analyzed with the gelatin zymography procedure (Heussen and Dowdle 1980). Briefly, conditioned media from organotypical brain slice cultures or cell cultures or whole slice cultures were mixed with sample buffer, and the samples were loaded on 7,5% SDS-PAGE containing 1% gelatin (table 2.3.). After electrophoresis (4°C, 90V), the gel was washed two times 15min in 2,5% Tx-100 washing solution, and incubated overnight in developing buffer. Next, the gel was stained for 30min in 0,5% Coomassie blue solution and then destained in 40% methanol and 10% acetic acid. The gel was wrapped between two foils of cellophane and dried in the gel dryer for 1h. Enzymatic activity resulted in gelatin degradation, which was directly visible as clear bands on the dark blue background. The surface of these clear bands directly correlated to the amount of the active gelatinase. The different gelatinase types were identified by their corresponding molecular weights.

	7,5%PAAG, 1%Gelatin	4% (loading) gel
dd H_2O	4,35ml	6,10ml
20mg/ml gelatin	0,5ml	-
30% Acrylamide	2,5ml	1,3ml
1,5M TRIS HCl, pH 8,8	2,5ml	-
0,5MTRIS HCl, pH 6,8	-	2,5ml
10% SDS	0,1ml	0,1ml
10% APS	0,1ml	0,1ml
TEMED	35µl	35µl

Table2.3. Composition of 1% Gelatin 7,5% SDS Polyacrylamide gels

2.2.13. Western blot

2.2.13.1. Sample preparation

The cells were quickly washed two times with ice-cold PBS and then all PBS was carefully removed. Sample buffer was applied to the cells (10µl/cm^2) and the cells were scraped with a rubber policeman. Cell lysates were collected in 1,5ml tube and incubated on ice for 15 min. Then, the samples were centrifuged at 13000rpm for 20min. The supernatant was collected and the pellet discarded. The protein concentration was determined with the BCA assay (see below). In all samples the protein concentration was equilibrated with sample buffer. Mercapto-ethanol (2%)

and glycerol (20%) was added to the samples and they were incubated at 95°C for 15min.

2.2.13.2. SDS PAGE

The gels were casted in glass plates. The composition of the gels is shown in Table 2.4. The gels were loaded with 10µl molecular weight marker and 20µl of each sample. Then the gel was run for 10min at 100V, than 70min at 150V.

Separating gel (lower gel)	
Lower gel buffer	2,5ml
Acrylamide	3,3ml
12% glycerol	4,17ml
10% APS	30µl
TEMED	10µl
Stacking gel (upper gel)	
Upper gel buffer	1,25ml
Acrylamide	0,750ml
12% glycerol	3,0ml
10% APS	30µl
TEMED	10µl

Table 2.4.Composition of 10% SDS Polyacrylamide gels

2.2.13. 3. Semi-dry trans blotting

The gels were carefully removed from the glass plates and equilibrated in transfer buffer for 10 min. Meanwhile, a PVDF membrane was activated by incubation in methanol for 5min, and equilibrated in transfer buffer for 5 min. Simultaneously blotting paper was incubated in transfer buffer for 5 min. Then, the moist blotting paper was placed on the lower electrode (anode), the PVDF membrane onto the blotting paper and then the separating gel onto the membrane and covered with another moist blotting paper. This sandwich was covered with the upper electrode (cathode) and electroblotted at 15V for 60min.

2.2.13.4. Immunoblotting

The membranes were first blocked with western blot blocking buffer for 30min. Then, the primary antibody was applied in blocking buffer ON at 4°C on the shaker. After that the membrane washed three times in TBST, and incubated with HRP conjugated secondary antibody for 1h at RT. After three washes for 20min in TBST, the ECL reagent was applied for 5min. The membrane was dried from excess ECL reagent and placed on the transparent foil. The membranes were placed in a film cassette, exposed to ECL-films (in the dark room) for a various time periods (e.g. 15s, 30s, 1min, 5min) and the films were developed in the film developing machine.

2.2.14. MT1-MMP activity assay

This ELISA based assay measures amounts of active MT1-MMP. It was performed according to manufacturers instructions in a 96 well plate. First, immobilized MT1-MMP monoclonal antibody was used to capture all forms of MT1-MMP from microglia cell lysates. Then, the pro-form of the detection enzyme (pro-urokinase) was added. To achive greater assay specificity, the producer had replaced the natural activation sequence in the pro-urokinase with an artificial sequence, which is only recognized by active MT1-MMP. Finally, the substrate was added and a change in color was measured at 405nm in microplate reader, indicating the amount of active MT1-MMP.

2.2.15. Statistical analysis

Statistical analyses were performed using SPSS software. Statistical significance was determined at the $p < 0.05$ level. The results are expressed as mean values± standard errors of the mean (SEM). Comparisons among the groups were performed with the Paired- Samples t Test and the Wilcoxon signed ranks test.