4. Discussion

4.1. Organotypical brain slice cultures are an in vitro experimental model which resembles important features of living brain

Since organotypical brain slice culture was introduced in 1991 as a new model for neuroscience by Stoppini (Stoppini, Buchs et al. 1991), it was used in more than 500 studies. The organotypical brain slice cultures were successfully used in a wide range of experiments: analysis of neuronal proliferation, apoptosis and migration in embryonic neocortex development (Haydar, Bambrick et al. 1999; Marin-Teva, Dusart et al. 2004); neuronal stem cell research (Schmidt-Kastner and Humpel 2002) and glioma cell invasion (Markovic, Glass et al. 2005). The report by Scheffler (Scheffler, Schmandt et al. 2003) nicely illustrates from where the properties and potentials of organotypical brain slice culture model derive . They showed not only that organotypical brain slice cultures are alive and electrophysiologicaly active (they recorded field potentials after stimulation of perforant path and Schaffer collaterals) after 33 days in vitro but also that grafted embryonic STEM cells into organotypical brain slice cultures were able to integrate into pre-existed astrocytic networks. These studies highlight the advantages of the organotypical brain slice culture system and indicate that it is the one culture model, which reflects the in vivo situation most closely. Here, all cellular constituents of the brain parenchyma like neurons, astrocytes, microglia, oligodendrocytes and endothelial cells are present and maintain the micro-architecture of the brain. Further, this system allows a large number of experimental repeats using only a small number of animals; it offers easy and fast handling and provides the possibility to bypass the BBB and to apply pharmacological compounds which normally can not penetrate the BBB (e.g. clodonate liposomes). Finally the organotypical brain slice cultures can be readily inspected by a microscope. Naturally, there is no cerebral blood flow and no infiltration by other peripherial blood-borne cells or factors like e.g. monocytes, T lymphocytes, leukocytes, antibodies and so on. However, this was an advantage of organotypical brain slice culture model because I wanted to observe the effects of microglia on gliomas without the contribution of peripheral monocytes and under exclusion of neoangiogenesis. To my knowledge this culture system represents the only possibility to dissect the contribution of microglia to glioma pathology under in vivo-like conditions.

4.2. A model for studying human gliomas

4.2.1. Organotypical brain slice culture is model of choice to study glioma invasion in vitro

The insidious infiltration of brain tumor cells into the surrounding normal brain makes total surgical removal of the tumor impossible, and therefore represents the major obstacle for successful therapy. Various model systems in vivo and in vitro have been developed to experimentally study brain tumor invasion. The in vivo models principle is to inject brain tumor cells into rodent brains or by implanting them subcutaneously and subsequently measure the tumor size. Besides, there are several in vitro models of cellular migration. In the scratch model one line is scratched into a confluent cell monolayer with a sterile needle, then the time for closure of the scratch mark is measured (Majack and Clowes 1984; Murugesan, Chisolm et al. 1993; Sarkar, Meinberg et al. 1996). In the Boyden chamber assay, cell migration between a first cell reservoir (starting point) and a second cell reservoir (end point), is quantified. The two compartments are separated by a membrane and the number of cells, which arrive in the second reservoir-after penetrating through the membrane are quantified as *migrating* cells (Albini, Iwamoto et al. 1987; Bae, Arand et al. 1993; Janiak, Hashmi et al. 1994). A modification of the Boyden chamber assay can be used to study cell *invasiveness* in vitro, i.e. cells need to invade through a layer of extracellular matrix (Matrigel), which is coating the membrane. Along with that there are several other matrix-degradation assays which are interesting to mention like time-lapse microscopy of the cultured cells or tissue explants in the Matrigel (Zhang, Kelly et al. 1998; Dai, Alt et al. 2005), which allows direct filming and observing of the cellular movements trough protein matrix or in situ zymography where the tissue sections are mounted onto microscope slide pre-coated with gelatine, thus allowing direct visualization of increased gelatinolitic activity (Nakamura, Ueno et al. 1999). The mentioned migration models are rapid, quantitative methods; many factors that influence invasion can be screened with these assays. Although their physiological relevance has occasionally been questioned, in in vitro models tumor cells are invading through rather artificial media and not through realistic in vivo situations e.g. they rather penetrate through plastic membranes or ECM proteins gels without the need to squeeze trough the narrow brain extracellular space. Organotypic co-culture models have been used as a suitable environment to study brain tumor invasion (Bjerkvig, Laerum et al. 1986; Bjerkvig, Tonnesen et al. 1990; Lund-

Johansen, Engebraaten et al. 1990; Pedersen, Ness et al. 1994). The organotypical brain slice cultures may be used in different fashions; implanting glioma cell aggregates into the organotypical brain slice cultures (3D interface), or by glioma cell aggregates sandwiched between two slice cultures (2D interface). These brain slice cultures involve viable brain tissues resembling
more accurately the in vivo situation than conventional in vitro systems, where single tumor cells invade into a nonviable matrix (Easty and Easty 1974; Nygaard, Pedersen et al. 1995). Ohnishi et al. (Ohnishi, Matsumura et al. 1998), used rat brain slices obtained from the hippocampus or cortical regions of 2-days-old rats and maintained the brain slices in culture at the interface between air and the culture medium. Similarly to the model described by Ohnishi et al. (Ohnishi, Matsumura et al. 1998), I used mouse brain tissue derived from 16 days old C57/BL6 mice to establish the organotypical brain slice culture model. The mice 16 days of age were used to combine optimal cell viability with optimal consistency with the brain tissue. Firstly the simple rule in slice culture is the younger the tissue is derived from the better since the viability of the cells during long period cultivation is better preserved, secondly it is easier to cut brain slices of that age and produce less tissue damage because tissue contains optimal amount of water and by that is easy to cut. Most importantly at this age the microglial development and maturation is finished (Streit 2001). Living brain tissue is an ideal matrix for studying malignant glioma cell invasion in vitro. In experimental tumor models and histopathological preparations, single-cell infiltration and micrometastasis have often been difficult to study because of a lack of suitable and sensitive markers that can discriminate individual tumor cells from the normal cell population. Recently, a number of vital dyes (Fast blue, Fluorogold, DiI, and DiO) and reporter genes (lac Z) have been used to study tumor cell migration in the brain (Andersson, Capala et al. 1992; Espinosa de los Monteros, Bernard et al. 1993; Lampson, Lampson et al. 1993; Marienhagen, Pedersen et al. 1994; Chicoine and Silbergeld 1995; Chicoine and Silbergeld 1995). GFP has been used to monitor gene expression and protein localization in living organisms, to infect neurons in the brain of frogs (Wu, Zou et al. 1995), or to visualize cancer invasion and metastasis (Chishima, Miyagi et al. 1997; Chishima, Miyagi et al. 1997; Deisboeck, Berens et al. 2001). GFP, a single peptide of 238 amino acids derived from the jellyfish A.Victoria, absorbs blue light and emits green light without the need for any cofactor or substrate. EGFP has a chromophore that is 35 times brighter than wildtype GFP. Study of the invasion of brain tumors in an organotypical brain

slice culture model system is more vital and reliable than other in vitro models. It resembles more closely the in vivo situation with regard to cell shape and cellular environment and can therefore determine the physiological behavior of the cells although it cannot completely replace the evaluation of brain tumor invasion in vivo. Limitations of the organotypical brain slice culture are abrogation of blood supply to the tissue, finite lifespan of the tissue in vitro, and lack of directed immune-responsiveness to the implanted tumor cells. I have used EGFP expressing glioma cells to visualize individual tumor cells which make it easy to determine the precise pattern of glioma invasion. This organotypical brain slice culture model supports the study of spatial dynamics of tumor cells. It is also very useful in understanding the biological dynamics of infiltrating tumor cells through the normal brain in vitro. Organotypical brain slice cultures were well preserved histologically for a complete experimental period –nine days (Fig. 3.2.) - which was enough for the evaluation of tumor invasion in this model system. It was possible to observe the invading tumor cells under a fluorescence or confocal microscope. Tumor spreading in the brain slice was evident even after four days (Fig. 3.5). After nine days the cytoarchitecture of the brain tissue remained viable and intact without showing histopathological signs of obvious necrosis.

In summary, the brain-slice model has several distinct advantages over the conventional basement membrane-based assays and the confrontational assay system for the purposes of studying glioma invasion. Most notably in the organotypical brain slice culture glioma invasion model, the cells of brain parenchyma are able to interact with glioma cells in contrast to other, mostly artificial, invasion assays which are cell free. In neurooncology there are well recognized interactions between glioma cells and brain parenchyma: glioma as a by-product of their metabolism release neurotoxic amounts of glutamate and by that induce neural death (Chung, Lyons et al. 2005); astrocytes are exploited by glioma cells to increase glioma invasiveness (Le, Besson et al. 2003); glioma cells migrate and proliferate at the brain vasculature (Farin, Suzuki et al. 2006); microglia in several recent reports is presented as a tumor promoting factor (Bettinger, Thanos et al. 2002; Platten, Kretz et al. 2003; Pollard 2004; Markovic, Glass et al. 2005). Currently the role of monocytes in cancer in general is reconsidered. Previously, the presence of monocytes or their differentiated successors in cancer surrounding-tumor associated macrophages (TAM) was considered as antigen presented cells of innate immune system and by their direct anti-tumorigenic response. But the recent experimental data show that the net effect

of TAMs which are present in human cancer is mostly pro-tumorigenic (Lewis and Murdoch 2005; Condeelis and Pollard 2006; Gordon and Freedman 2006). The TAMs can release growth and survival factors, over-express metalloproteinases or down-regulate antibody mediated phagocytosis, and by that support cancer proliferation, survival and metastasis.

In the present study, EGFP gene-transfected rodent glioma cells were used to visualize tumor progression through the mouse brain in vitro. The model system I developed can provide reliable and important information in elucidating the role of microglia in modifying brain tumor invasion and progression.

4.2.2. Gliomas in organotypical brain slice cultures are sharing features with human gliomas

Even low-grade cerebral gliomas are infiltrative, whereas malignant gliomas are widely invasive (Lampert, Machein et al. 1998). They spread locally through extensions of infiltrating tumor cells in normal brain or, more rarely, through cerebral spinal fluid. The single-cell migration through the brain parenchyma at the tumor periphery is more common than spreading in one tumor mass (Kleihues 1996). The microglia are major immune cells which surround the human gliomas (Strik, Stoll et al. 2004). Moreover, their number is in direct correlation to the malignancy grade of gliomas (Roggendorf, Strupp et al. 1996). In comparison, I found glioma cells at the invasive edge of the tumors with the stretched morphology reminiscent of migratory cells that progressed into the parenchyma either as single cells or as clusters of cells. The invading glioma cell clusters and individual glioma cells were partly associated with blood vessels. Besides that, single tumor cells and tumor cell aggregates, which displayed a migratory phenotype and which were not found in proximity to vessels, are also detected. Therefore, the invasion pattern of glioma cell lines in the organotypical brain slice culture model exhibits some similarities to gliomas in humans, such as the diffuse invasion of single cells through the brain parenchyma (Guillamo, Lisovoski et al. 2001). Additionally observed was the progression of glioma cell-clusters along blood vessels, a type of invasion that is attributed as typical for rodent glioma cells. Moreover, a close association of microglial cells with the infiltrating tumor cells was always observed, a characteristic of human pathology, as microglia infiltrate the glioma cell mass (Badie and Schartner 2001; Strik, Stoll et al. 2004). The amount of microglial cells found within human gliomas can vary between

approximately 10% to approximately 34% (Badie and Schartner 2000; Strik, Stoll et al. 2004). However, in some human gliomas the microglia primarily associates with the glioma cells at the invasion edge of the tumors (Roggendorf, Strupp et al. 1996). Therefore, even lower microglia cell numbers may efficiently promote glioma invasion. The organotypical brain slice culture model, which mimics in vivo or clinical situations, makes it possible to evaluate the behavior of individual cells in primary brain tumors under physiological conditions.

4.3. Microglia depletion

I applied clodronate filled liposomes onto cultured brain slices to selectively induce cell death in microglia. Subsequently, the altered invasiveness in the experimental glioma organotypical brain slice culture model was measured. The clodronate-liposomes are an efficient tool to deplete microglia in cultured brain slices. Clodronate liposomes induced cell death in monocytes and tissue macrophages in vivo in experimental animals (Van Rooijen and Sanders 1994). Clodonate impedes the mevalonate pathway of cholesterol synthesis which heads to a lack of prenylation of small GTPases that are essential for microglia functions and survival, finally resulting in microglia cell death (Reszka and Rodan 2004). I established a procedure where microglia can be selectively depleted in organotypical brain slice cultures (Fig. 3.1.). It is based on the exclusive phagocytosis of liposomes by microglial cells in the brain and the cytotoxic action of clodronate. The lack of a BBB in this in vitro model allows the microglia free access to clodronate-filled liposomes at the slice surfaces. The subsequent phagocytosis of clodronate filled liposomes at the organotypical brain slice culture surfaces by microglia leads to selective microglia cell death (Fig. 3.1.; (Markovic, Glass et al. 2005). Moreover, this approach has been previously used to deplete microglia in cultured cerebellar slices (Marin-Teva, Dusart et al. 2004). These authors reported selective microglial depletion using clodronate liposomes in cereberal brain slice cultures obtained from P3 mice (Marin-Teva, Dusart et al. 2004). As a further control I used PBS-filled liposomes. Under this paradigm microglia exhibited an amoeboid morphology indicating a certain level of activation, presumably due to the phagocytosis process per se (Van Rooijen and Sanders 1994). Closer inspection of the slices indicated that microglia not only became activated but also migrated preferentially to the surface of the slices where the liposomes were accumulated and thereby led to a decrease in microglia density in the center of the

slice. Whereas the clodronate-liposomes treated slices were entirely microglia free, the PBS-liposomes treated slices were regionally depleted (partially free of microglia). In other words, the PBS-liposome treatment of the organotypical brain slice culture did not produce an unbiased "control" group, since it did not represent microglial in vivo distribution (Nimmerjahn, Kirchhoff et al. 2005). Moreover, I found an effect on glioma invasion in such PBS liposomes treated organotypical brain slice cultures, the rate of glioma invasiveness was in between the values of clodronate liposomes and control groups (Fig. 3.10.). However, in normally cultured, not liposomes-treated organotypical brain slice cultures, the regular distribution of microglia was observed (Fig. 3.1.) and represented the control group. Nonetheless, amoeboid morphology was to a great extent observed in this group as well, again indicating a certain level of microglia activation. Clodronate liposomes were not cytotoxic for glioma cells, as demonstrated in Fig. 3.2. The viability remained the same as compared to the control organotypical brain slice cultures indicating no influence of the clodronate liposomes treatment on slice viability. Therefore, untreated organotypical brain slice culture was used as the relevant (unbiased) control group, since only these preparations contain normally distributed microglia.

4.3.1. Microglia

It was tested if a well known activator of microglia, namely the pyrogenic lipopolysaccharides of the Gram negative bacterial membrane (LPS), can enhance or otherwise modify the pro-tumorigenic effects of microglia in glioma infiltrate. A series of experiments showed that LPS treatment does not influence glioma invasiveness in intact organotypical brain slice cultures or in microglia organotypical brain slice cultures (Fig. 3.10.). This indicates that full activation of microglia does not play an additional role in the microglial effect on glioma migration, probably because microglial activation beyond certain point does not have further effects on glioma or because glioma may attenuate the microglial activation by their well known immunomodulatory effects (Badie and Schartner 2001). In human gliomas the tumor-surrounding microglia appears activated since it exhibit its amoeboid morphology (Graeber, Scheithauer et al. 2002), however, for full activation certain other criteria need to be fulfilled like expression of MHC I and II, CD4 and CD8 molecules (Streit 2005) or display altered electrophysilogical properties (Farber and Kettenmann 2005).

4.3.2. Astrocytes

Astrocytes express low amounts of GFAP in the organotypical brain slice culture in normal or in PBS liposome-culturing conditions (Fig. 3.4.). However, when the clodronate liposomes were applied to the organotypical brain slice culture, astrocytes strongly up-regulate GFAP expression (Fig. 3.4.). The astrocytes are activated as a physiological reaction to the surrounding microglia death. Astrocytes do not react on liposomes themselves since PBS-filled liposomes did not have a great effect on GFAP expression. Interestingly, further culturing of the clodronate liposome treated organotypical brain slice cultures slowly decreased the astrocytic GFAP expression. After 72h of normal culturing conditions they again acquired the resting state as in the control organotypical brain slice cultures (Fig. 3.4.). Both the expression of GFAP and the morphology were very similar in the control and clodronate-treated organotypical brain slice cultures 96h after preparation (Fig. 3.4. and Fig. 3.5.). Most notably, the long and fine filamentous astrocytic processes were unchanged by clodronate liposomes treatment (Fig. 3.4.; insert).

4.3.3. Endothelium

Endothelial cells are present even after extensive culturing periods in the organotypical brain slice cultures, as is illustrated by IL-B4 labelling (Fig. 3.2.) and Von Willebrand factor (vWf) antibody immunohistochemistry (Fig. 3.7.). Interestingly, the endothelial cells in the organotypical brain slice cultures maintained the morphology of blood vessels during the experimental period (nine days in culture (Fig. 3.7.). As reported previously (Farin, Suzuki et al. 2006) they were able also to attract and associate with glioma cells (Fig. 3.7., insert).

4.3.4. Neurons

Neurons in the middle of the organotypical brain slice culture remain largely intact and display no pathological morphology (Fig. 3.5.). Only neurons at the surface of the brain slices show some budding, since in this plane processes of individual neurons had been cut during slice preparation.

4.4. Microglia promote tumor invasion.

As an indicator of invasion I measured the migration distances traveled by individual glioma cells under the different experimental paradigms. Directly after injection, the tumor cells were spread about 250µm around the injection site (Fig. 3.6.A-D). Throughout the first two days the tumor remained almost invariably within a diameter of about 500µm from the injection site and only a few isolated cells occasionally migrated beyond this border. After four or five days, however, many cells had migrated more than 500µm, with a maximum distance of 1,5mm (Fig. 3.8.). To define a subpopulation of invasive glioma cells I arbitrarily defined those cells which remained confined within 500µm from the start site as stationary, cells which migrated beyond that border I regarded as invasive. Results from the migratory assays indicate that the density of microglia in the tissue regulates glioma invasion: (1) Glioma in microglia-depleted organotypical brain slice cultures show the lowest invasive potential (Fig. 3.8.). (2) Addition of microglia to microglia depleted slices restores the invasive potential to the level of control organotypical brain slice cultures (Fig. 3.9.). ma migration by partially or regionally (4) Co-injection of cells with microglia into the control organotypical brain slice cultures led to the highest invasion activity (Fig. 3.9.). The last point may be explained by two independent effects. Firstly and most importantly, the amount of microglia is directly and strongly elevated within the tumor, up to levels which may not be reached without addition of microglia. Secondly, the microglia under control conditions may need some time to initiate a full blown response and to arrive at the lesion and by that the pro-invasive effect in glioma is initiated later. Roggendorf et al described in a neuropathological study that the number of microglia directly correlates to the grade of invasiveness in human gliomas (Roggendorf, Strupp et al. 1996). Additionally, I discovered that glioma invasive potential increases with the amount of microglia infiltrating experimental tumors. This suggests the presence of a vicious circle: on the one hand more microglia is attracted towards glioma (Roggendorf, Strupp et al. 1996) and on the other hand there is more invasiveness of glioma cells because more microglia gets associated with the gliomas (Pollard 2004; Markovic, Glass et al. 2005). The correlation presented in this work raises the question whether pathologically relevant aspects of glioma malignancy can be studied in isolation, ignoring the surrounding cells. Perhaps they are malignant by their ability to exploit reactive cells from their immediate vicinity to support their own invasion like e.g. microglia, (Markovic, Glass et al. 2005), astrocytes, (Le, Besson et al. 2003) and endothelial cells (Farin,

Suzuki et al. 2006). These results are going along with the report from Bettinger et al., who used the Boyden chamber migratory assay and showed that microglia promote glioma migration (Bettinger, Thanos et al. 2002). Hence, they demonstrated that microglia cultured in a lower reservoir of the Boyden chamber were able to chemoattract glioma cells to migrate through 8μm polycarbonate membrane by a factor released from microglia. However, their experimental model neglected the presence of ECM proteins and whole brain tissue in the glioma surrounding. Bettinger and colleagues evaluated only chemoattractive properties of microglia towards glioma and not the complete microglia-glioma interaction in the brain. As I will discuss later, the tumor-promoting effect of microglia occurs after microglia-glioma interaction and is mediated by the membrane type I matrix metalloprotease (MT1-MMP). Thus, microglia must be in contact or close proximity to glioma cells to exhibit pro-invasive effects.

4.5. MMP-2 activity in gliomas

Glioblastomas are tumors with high invasiveness (Holland 2000) and the degradation of ECM is a prerequisite for the invasive potential (Matrisian 1992). Significant evidence has accumulated that directly implicates members of the MMP gene family in this process. MMP-2 (Gelatinase A) is one of the most abundant proteases in human brain tumors and its activity correlates with WHO grade of gliomas (Lampert, Machein et al. 1998). As presented in Fig. 3.11. MMP-2 is secreted as a non active zymogen (pro-MMP-2) and in the extra cellular space pro-MMP-2 can be activated by proteolytical cleavage of the pro-enzyme part into fully active MMP-2. Because of the important role of active MMP-2 in invasive behaviour of gliomas, I measured MMP-2 activity in glioma bearing organotypical brain slice cultures using gelatine zymography. This assay detected a robust MMP-2 activity in the organotypical brain slice cultures inoculated with GL261. Interestingly, the highest MMP-2 activity was measured in slices co-inoculated with GL261 cells and microglia (Fig. 3. 11. A-B) whereas the lowest activity of MMP-2 was measured in microglia depleted slices. When I assayed the activity of MMP-2 released into the slice-culture supernatant, I detected again that an increased abundance of microglia within glioma yielded high MMP-2 activity. As expected for a released enzyme, MMP-2 activity generally produced stronger bands in zymograms from supernatants than from tissue samples.

These results indicate that the abundance of microglia directly correlates with MMP-2 activity in the organotypical brain slice culture glioma model.

How are microglia able to stimulate the MMP-2 release from the organotypical brain slice culture? Glioma readily secrete pro-MMP-2 and subsequently need to activate the enzyme on the outer plasma membrane (Deryugina, Bourdon et al. 1997). Microglia can also express pro-MMP-2 under pathological conditions and subsequently activate it (Cross and Woodroofe 1999).The possibilities are that microglia themselves secret pro-MMP-2 in the organotypical brain slice culture, that microglia somehow stimulates GL261 to liberate more MMP-2 or that glioma release large amounts of the inactive pro-form of MMP-2 which gets activated much more efficiently in the presence of microglia. Further cell culture experiments revealed that the net-activity of MMPs was increased, when microglia liberated active MMP-2 after incubation with glioma conditioned medium (Fig. 3.11.C). This experiment narrowed down the number of possible mechanisms to only two possibilities, either that some factor from GCM stimulates microglia to release MMP-2 de novo, or alternatively that GL261 cells secrete mostly pro-MMP-2, which is then processed and activated by microglia. An additional series of cell culture experiments indicated that stimulated microglia do not increase their pro-MMP-2 expression (Fig. 3.12.), but produce more MT1-MMP (Fig. 3.13, 3.14, 3.15 and 3.16). The latter proteinase is the single most important activator of pro-MMP-2. In conclusion I favour a model in which glioma secrete great amounts of pro-MMP-2, but can only activate a fraction of the pro-enzyme. However, they co-secrete a factor that stimulates the expression of MT1-MMP in the surrounding microglia which then finally activates the excess of the pro-MMP-2 originally liberated from the tumor. Thereby microglia facilitates glioma invasion.

4.6. MT1-MMP is expressed predominantly in microglia in mouse gliomas

An in vivo mouse glioma model revealed that Iba-1 positive cells of normal brain express MT1-MMP only at a very low level whereas brain tumor surrounding microglia strongly expresses MT1-MMP (Fig. 3.13.). Immunohistochemistry of these sections disclosed that the amount of MT1-MMP labelling specifically increased in microglia in immediate surrounding of the glioma. Colabelling experiments with antibodies for MT1-MMP and Iba-1 revealed that both antigens were expressed in the same cell population. This colabelling was most intense directly around the

experimental gliomas. Hence, the leading cell type showing MT1-MMP over-expression in glioma infiltrates is microglia. Interestingly, Bar-Or and collegues reported similar findings when they analysed inflammation-activated monocytes from multiple sclerosis patients (Bar-Or, Nuttall et al. 2003). This group detected by FACS analysis an increased expression of MT1-MMP in human blood monocytes during the acute phase of multiple sclerosis. Hence, pro-inflamatory mediators may trigger MT1-MMP over-expresion.

4.7. MT1-MMP is up-regulated on microglia when stimulated with GL261 conditioned medium (GCM)

De novo synthesis of MT1-MMP mRNA indicates that certain factor(s) from GCM stimulate MT1-MMP production (Fig. 3.14). Already three hours after GCM stimulation the amount of MT1-MMP mRNA in microglia is increased in comparison to the basal MT1-MMP mRNA production. Six hours after stimulation the MT1-MMP mRNA levels reach a peak. Western blots from microglial cultures stimulated with GCM revealed that also the protein level of MT1-MMP is increased, supporting the mRNA results. The protease MT1-MMP can undergo different auto proteolytical reactions predominantly depending on the concentration of its inhibitor, tissue inhibitor of metalloprotease-2 (TIMP-2; (Deryugina, Bourdon et al. 1997). The first proteolytical reaction cleaving the inactive zymogen (~60kDa) into the active form (~57kDa) occurs in the Golgi-networks just before the insertion of the active form into the plasma membrane (Sato, Takino et al. 1996; Osenkowski, Toth et al. 2004). Then, the active form with its catalytic domain facing towards the extracellular space is able to cleave extracellular substrates like ECM proteins, activate pro-MMP-2 by cleavage of pro-enzyme part (Pei and Weiss 1996; d'Ortho, Will et al. 1997; Ohuchi, Imai et al. 1997) or to undergo an autocatalytic self-degradation (Toth, Hernandez-Barrantes et al. 2002). Examination of the profile of MT1-MMP forms in cell extracts showed that in addition to the active species, MT1-MMP is also found as a series of variants ranging from ~44 to 40kDa (Lehti, Lohi et al. 1998; Hernandez-Barrantes, Bernardo et al. 2002). In fact, next to the presence of the 57kDa molecule, the 44kDa product is an additional indicator of the increased turnover of the active enzyme (Cowell, Knauper et al. 1998; Lehti, Lohi et al. 1998). Furthermore, I quantified the increase in the amount of active MT1-MMP using a MT1-MMP activity assay (Fig. 3.16.). This assay disclosed that six hour GCM treatment increased the MT1-MMP

activity in microglia three fold. Taken together, the dynamics of MT1-MMP show that gliomas are able to induce an up-regulation of MT1-MMP in microglia thereby allowing a stronger activation of glioma derived pro-MMP-2.

4.8. GL261 conditioned medium (GCM) up-regulates MT1-MMP in microglia via the p38 MAPK pathway

Recent reports indicate that MT1-MMP expression is regulated through mitogen activated protein kinase (MAPK) pathways (Munshi, Wu et al. 2004; Boyd, Doyle et al. 2005). These authors demonstrate that a distinct branch of MAPK pathway is activated, e.g. p38 MAPK is activated but not ERK1/2 (Munshi, Wu et al. 2004). To test whether specifically p38 MAPK is involved in signal transduction in microglia leading to over-expression of MT1-MMP after stimulation with GCM a specific blocker of p38 MAPK (SB202190) was used. And indeed, as assessed by RT-PCR and Western blot, the over-expression of MT1-MMP in GCM stimulated microglia was blocked when these cells were treated with SB202190 (Fig. 3. 14, 15 and 16). After treatment with SB202190 some residual 44kDa fragment was detectable but in absence of the 57kDa species the 44kDa molecule only indicates the degradation product of MT1-MMP that was expressed prior to treatment with the MAPK blocker. In summary, this demonstrates that de novo production of MT1-MMP is inhibited whereas the proteolitic selfdegradation of MT1-MMP is not affected. Additionally, this explains why after cykloheximide treatment there was still activation of pro-MMP-2 released from glioma cells (Fig. 3.12.). Hence, MT1-MMP synthesized before cykloheximide treatment activates extracellular pro-MMP-2.

Supporting these results, unpublished data from the Kaminska lab (personal communication by Prof. B. Kaminska) indicate that GCM differentially activates branches of the MAPK pathway in primary microglia by phosphorylation of p38 MAPK (data not shown). They demonstrated the activation of the p38 MAPK and showed that ERK1/2 is inactive at three and six hours after glioma conditioned medium (GCM) stimulation of primary rat microglia. Furthermore, they examined the ability of GCM to stimulate the phoshorylation of the p38 MAPK downstream target mitogen activated protein kinase- activated protein kinase two (MAPKAPK-2). Along with an increased level of p38 MAPK phosphorylation the amount of phoshorylated MAPKAPK-2 (at Threonine 334) peaked at six hours after GCM treatment.

Interestingly, even though p38 MAPK pathway was activated after GCM stimulation, the ERK1/2 phosphorylation was absent three and six hours after GCM treatment.

In summary, the factor released from GL261 cells selectively activates the p38 MAPK pathway which in turn regulates increased expression of MT1-MMP in microglia. Importantly, the blockage of p38 MAPK activation by the specific blocker SB202190 abrogates expression of MT1-MMP, disclosing p38 MAPK as a mediator of MT1-MMP overexpression. Downregulating the enormous capacity of glioma cells to invade would be fundamental to any successful therapy of these tumors. The central new finding of my studies was that this invasion promoting effect needs to be abrogated in both the parenchymal cells (i.e. the microglia) and the tumor cells.