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Experimental studies in brain tumours

- with special regard to multidrug resistance and the ErbB - family

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Till
mina älskade pojkar!
Jacob, Johan, Jimmy
Staffan

HÖGFALA

Dem söm sätt nesan opp i verä
å tro sä vara na stort
dem ha bra litä för besverä
Dä könnä likä gött fo bli ogjort,
för dä e ju sä ve människän.
All kom tommom å uttan ´n trå.
Å all ske fara klelös igen,
nanting var å en tord ha reda på!

Ur
För i ti´n å nu
-Smått och gott på vilhelminamål
Av Yngve Hellquist, 1997

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ABSTRACT

Primary brain tumours, and especially the most common form malignant gliomas, usually display a pronounced resistance to other treatment modalities when surgery fails to cure. Growth factors, such as EGF and its receptor, frequently amplified and overexpressed in malignant gliomas, and factors associated with multidrug resistance have been suggested to at least partially explain the poor outcome. The aim of this thesis was to characterise factors in primary brain tumours associated with the development of resistance with focus on the epidermal growth factor receptor (ErbB) family, and multidrug resistance (MDR).

Influences of irradiation on the expression and activity of P-glycoprotein (Pgp) in malignant gliomas was evaluated. The effects showed that irradiation increased the efflux activity of Pgp in rat brain vascular endothelial cells, but not in glioma cells. In the intracranial BT4C glioma model, Pgp was detected in the capillary endothelium in the tumour tissue but not in glioma cells.

Expression of several factors coupled to MDR (Pgp, MRP1, LRP, and MGMT) in primary brain tumours were analysed and correlated to clinical data. In gliomas, Pgp and MRP1 were predominantly observed in capillary endothelium and in scattered tumour cells, whereas LRP occurred only in tumour cells. In meningiomas, expression of the analysed markers was demonstrated in the capillary endothelium, with a higher expression of Pgp and MRP1 in transitional compared to meningothelial meningiomas. A pronounced expression of MGMT was found independently of the histopathological grade or tumour type. Survival analysis indicated a shorter overall survival for patients suffering from low-grade gliomas with high expression of Pgp.

To explore the importance of the epidermal growth factor receptor (EGFR), expression levels of the family members (EGFR, ErbB2-4) were analysed and their relations to various clinical parameters were evaluated in gliomas and meningiomas. In gliomas, the highest EGFR expression was observed in high-grade tumours, while ErbB4 expression was most pronounced in low-grade tumours. In meningiomas, expression of EGFR, ErbB2, and ErbB4 was observed in the majority of the tumours. An intriguing observation in low-grade gliomas was a significantly decreased overall survival for patients with high EGFR protein expression.

The effects of different time schedules for administration of the selective EGFR inhibitor ZD1839 in relation to irradiation of glioma cells were analysed. The analyses showed a heterogeneity in the cytotoxic effects of ZD1839 between cell lines, and it was obvious that some of the cell lines showed sensitivity to ZD1839 despite no or low expression of EGFR. The study also demonstrated the importance of timing of ZD1839 administration when this agent is combined with irradiation.

In conclusion, in order to enhance the efficacy of radiotherapy by various drugs in malignant gliomas it may be essential to inhibit drug efflux activity in endothelial cells and to deliver drugs in an optimal timing in relation to radiotherapy. The heterogeneity in expression of drug resistance markers, as well as the ErbB family reflects the complexity in classification of primary brain tumours, and indicates that subgroups of patients with low-grade gliomas expressing Pgp and EGFR might benefit from more aggressive and individualised treatment.

Keyword: glioma, meningioma, endothelium, MDR, Pgp, MRP1, LRP, MGMT, EGFR, ErbB2, ErbB3, ErbB4.

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numbers.

I. Rapid induction of long-lasting drug efflux activity in brain vascular endothelial cells but not malignant glioma following irradiation.
U. Andersson, K. Grankvist, A.T. Bergenheim, P. Behnam-Motlagh, H. Hedman, R. Henriksson. Medical Oncology, 19(1): 1-9, 2002.

II. Heterogeneity in the expression of markers for drug resistance in brain tumors.
U. Andersson, B. Malmer, A.T. Bergenheim, T. Brännström, R. Henriksson. Clinical Neuropathology, 23: 21-27, 2004.

III. Epidermal growth factor receptor family (EGFR, ErbB 2-4) in gliomas and meningiomas.
U. Andersson, D. Guo, B. Malmer, A.T. Bergenheim, T. Brännström, H. Hedman, R. Henriksson. Acta Neuropathologica, 108: 135-142, 2004.

IV. Treatment schedule is of importance when ZD1839 is combined with irradiation of glioma and endothelial cells *in vitro*.
U. Andersson, D. Johansson, P. Behnam-Motlagh, M. Johansson, B. Malmer. Manuscript.

ABBREVIATIONS

ABC	ATP-binding cassette
Akt	v-akt murine thymoma viral oncogene homolog
ATP	Adenosin triphosphate
BBB	Blood-brain barrier
bFGF	basic fibroblast growth factor
CDKN2A	Cyclin-dependent kinase inhibitor-2A
CNS	Central nervous system
Ct	Treshold cycle
DNA	Deoxyribonucleotide acid
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptor
ErbB	v-erb-b erythroblastic leukemia viral oncogene homolog
Erk	Extracellular signal regulated kinase receptor
FGFR	Fibroblast growth factor receptor
GBM	Glioblastoma multiforme
IB	Immunoblot analysis
IHC	Immunohistochemistry
HB-EGF	Heparin binding epidermal growth factor
HER	Human epidermal growth factor receptor
KDa	Kilo Dalton
LOH	Loss of heterozygosity
LRIG	Leucine-rich repeats and immunoglobulin-like domains
LRP	Lung resistance protein
MAPK	Mitogen activated protein kinase
Mdm-2	Murine double minute-2
MDR	Multidrug resistance
MGMT	O^6 methylguanine-DNA methyltransferase
MRP1	Multidrug resistance protein-1
MVP	Major vault protein
NF2	Neurofibromatosis, type 2
NRG	Neuregulin
PDGF	Platelet derived growth factor
Pgp	P-glycoprotein
PI3-kinase	Phosphatidyl inositol 3 kinase
PLC-γ	Phospholipase C
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase-polymerase chain reaction
TGF-α	Transforming growth factor- α
VEGF	Vascular endothelial growth factor

INTRODUCTION

GLIOMAS

Epidemiology

Primary intracranial tumours have their origin within the brain or the meninges, and secondary tumours, i.e. metastases, are spread from primary tumours located outside the central nervous system (CNS). The intracranial tumours are subdivided into malignant tumours with infiltrative growth, and benign tumours with restricted local growth. The benign tumours can, however, because of their location within the skull cause severe and life-threatening symptoms.

The most frequent primary brain tumours are malignant gliomas, which could be divided into astrocytomas, oligodendrogliomas, and oligoastrocytomas. The incidence of gliomas is about 6,0 per 100,000 (Lonn et al. 2004). They usually have an infiltrative type of growth, which makes total surgical resection impossible. Low-grade gliomas affect mainly young adults (Behin et al. 2003), whereas the incidence of high-grade gliomas is higher in elderly people.
With combined surgery and radiotherapy the 5 years survival of patients with low-grade glioma (grade II) is about 50%. Patients with high-grade glioma have an extremely poor prognosis. In these cases, surgery and radiotherapy may only prolong the expected survival from 5 to approximately 12 months (Bergenheim & Henriksson 1998; Henriksson et al. 1998). Recent reports have shown that oligodendrogliomas with allelic losses on chromosome arms 1p and 19q are significantly associated with both chemosensitivity and longer recurrence-free survival (Cairncross et al. 1998; Smith et al. 2000).

Etiology

The only established risk factors are ionizing irradiation (Little et al. 1998, Ron et al. 1998) and hereditary syndromes (Bondy et al. 1994; Inskip et al. 1995; Wrensch et al. 1997). There is some evidence that persons in certain occupations have a higher risk than others to develop brain tumours. Occupations reported to be associated with brain tumours include electrical (Cocco et al. 1998), petrochemical workers (Waxweiler et al. 1983; Bertazzi et al. 1989; Divine et al. 1999), and farmers (Khuder et al. 1998). However, the etiology of brain tumours is not strongly associated with any kind of exposure.

There are some hereditary syndromes, Li Fraumeni and Turcot, associated with an increased frequency of gliomas, indicating a genetic etiology in some cases (Li & Fraumeni 1969, Stevens & Flanagan 1986). With respect to heredity, Malmer et al. (1999) reported a family aggregation in first-degree relatives of malignant glioma in northern Sweden. In a Danish cancer incidence study of about 420,000 cellular phone users, no association between the use of cellular phones and malignant brain tumours (Johansen et al. 2001) was found. This finding was also supported in a recent Swedish study (Lonn et al. 2005). Contradictory, other studies have shown that use of a cellular phone yielded significantly increased risk for malignant brain tumours (e.g. Hardell et al. 2002).

Classification

The classification of glial tumours is difficult because these tumours are very heterogeneous and several classification systems such as, the Kernohan grading system (Kernohan et al. 1949, and the St. Anne/Mayo grading system (Daumas-Duport et al. 1988) have been used.

Nowadays, the WHO classification system is recommended. This classification is based on characterisation of the presumed cellular origin of the tumour and the histopathological grade of aggressiveness. Significant indicators of aggressive behaviour in gliomas include nuclear atypia, mitototic activity, cellularity, vascular proliferation, and necrosis. Based on the presence of these indicators, the grading system divides gliomas into four different grades WHO I-IV (Table 1).

Table 1. Comparison of the World Health Organisation, Kernohan, and St Anne/Mayo grading systems of astrocytomas.

WHO designation	WHO grade	Kernohan grade	St Anne/Mayo grade	St Anne/Mayo Histological criteria
Pilocytic astrocytoma	I	I	excluded	-
Diffuse astrocytoma	II	I, II	Astrocytoma grade 2	One criterion: usually nuclear atypia
Anaplastic astrocytoma	III	II, III	Astrocytoma grade 3	Two criteria: usually nuclear atypia and mitotic activity
Glioblastoma multiforme (GBM)	IV	III, IV	Astrocytoma grade 4	Three criteria: nuclear atypia, mitoses, endothelial proliferation and/or necrosis

Pilocytic astrocytomas correspond to WHO grade I, and diffuse astrocytomas to grade II, while anaplastic astrocytomas correspond to WHO grade III, and glioblastoma multiforme is classified as grade IV (Kleihues & Cavanee 2000).

The WHO classification for oligodendrogliomas and mixed oligoastrocytomas includes two grades, low-grade (grade II) and anaplastic (grade III), but the validity of the grading criteria remains debatable, and evidence is accumulating that a third group should be added, consisting of glioblastomas with an oligodendroglial component (He et al. 2001). Some tumours previously classified as astrocytomas are now being categorised as oligodendrogliomas or

mixed oligoastrocytomas (Daumas-Duport et al. 1997; Fortin et al. 1999; Burger et al. 2002).

It has to be emphasised that there are some limitations to be aware of when using the WHO classification and other grading systems. The systems are somewhat subjective since they are based on visual criteria only, which allow considerable observer variation in the diagnosis (Coons et al. 1997; Giannini et al. 2001).

Biology of astrocytomas

The growth patterns of gliomas vary with tumour grade. In general, glioma cells tend to migrate along white matter tracts and tumour cells may infiltrate parts of the brain remote from the primary location.

Low-grade astrocytomas consist of two major tumour types, pilocytic astrocytomas, and diffuse astrocytomas. Pilocytic astrocytomas are slowly growing and non-invasive, often occurring in children and young adults. Diffuse astrocytomas, on the other hand, are characterised by a high degree of cellular differentiation, slow growth, and diffuse infiltration of neighbouring brain structures. These diffuse lesions often affect young adults and have an intrinsic tendency for malignant progression to anaplastic astrocytomas, and, ultimately to glioblastomas.

High-grade astrocytomas grow with an expansive component and necrotic areas in the centre of the tumour are common. Glioblastomas are divided into primary and secondary gliomas. Primary glioblastomas are the de novo formation of glioblastomas without clinical or histological evidence that it has originated from low-grade tumours, while secondary glioblastomas arise from low-grade astrocytomas through stepwise genetic alterations. Overexpression of epidermal

growth factor receptor (EGFR), Mdm-2, p16 deletions, and PTEN mutations are coupled to primary glioblastomas, whereas p53 mutations are mainly associated with secondary glioblastomas (Kleihues et al. 1997) (Figure 1).

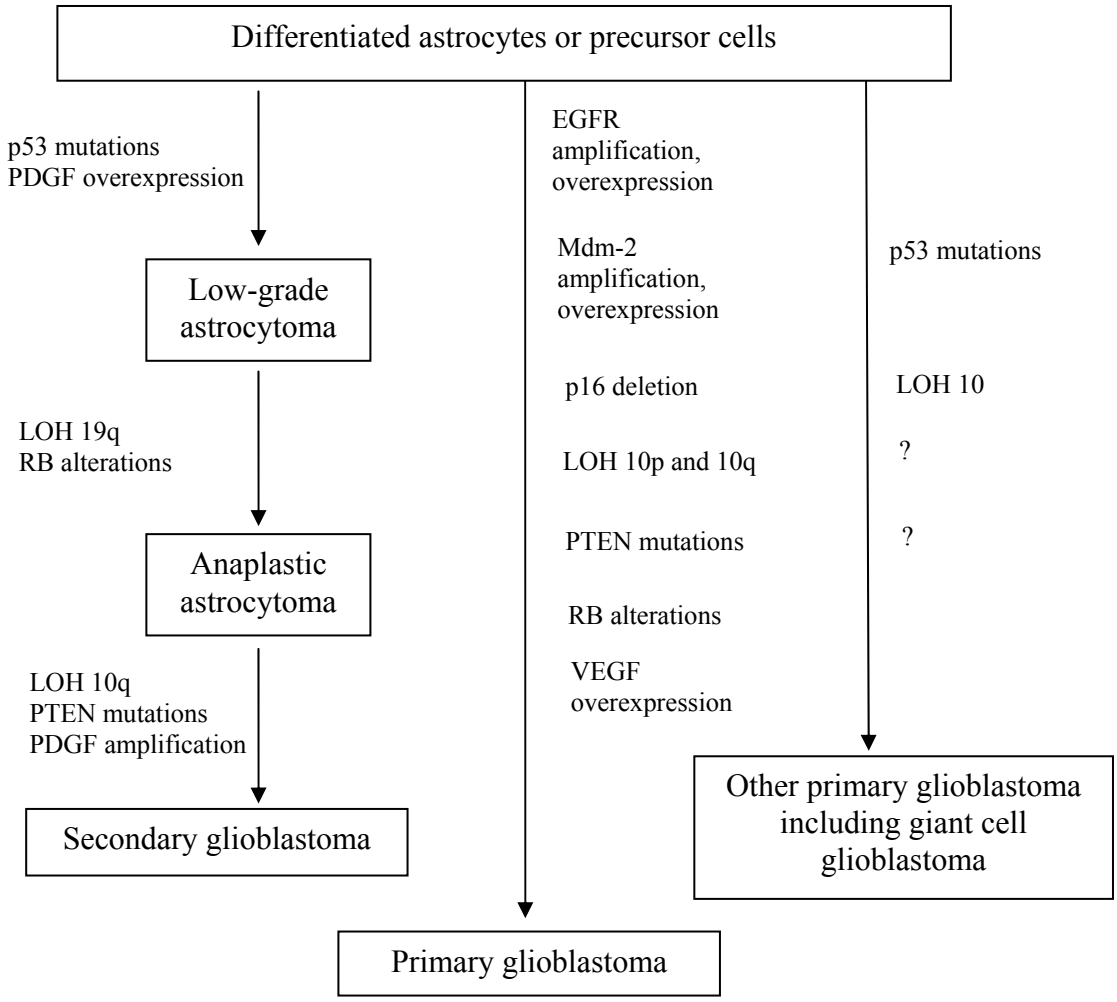

Figure 1. A model for possible steps in glioma progression. LOH=loss of heterozygosity. Modified from Kleihues & Cavanee 2000; Ohgaki et al. 2004.

Biology of oligodendrogliomas

Low-grade oligodendrogliomas (grade II) are slowly growing neoplasms and appear macroscopically to be quite well defined masses that often manifest after several years of preoperative epileptic seizures. The prognosis is more favourable compared with low-grade astrocytomas (grade II) (Engelhard et al. 2002). Anaplastic oligodendrogliomas appear de novo or sometimes results from the progression of low-grade oligodendrogliomas. This high-grade tumour is demonstrated as a contrast-enhancing heterogeneous mass, frequently with cystic components, calcifications, or necrosis.

Anaplastic oligodendrogliomas (grade III) often respond favourably to chemotherapy. Oligodendrogliomas represent the first type of brain tumours for which specific genetic alterations and immunohistochemical findings have significant prognostic value, and may even indicate the likelihood of response to chemotherapy. To date, the most important of these seems to be loss of 1p and 19q (Engelhard et al. 2002).

Treatment

Today the current treatment for adult malignant gliomas is based mainly on surgery, radiotherapy and in some cases chemotherapy. Although the surgical and radiological treatments have improved in recent years, their impact on patient survival is limited. Surgery is not a curative treatment, but it is still a keystone in the treatment of these tumours.

Malignant gliomas are relatively resistant to irradiation compared to many other tumours (Yang et al. 1990), which could be due to several factors. It is clear that brain tumours contain extensive regions in which the tumour cells are subjected to non-physiological levels of hypoxia. Hypoxia is well known for its negative

influence on the outcome of radiotherapy, since hypoxic cells are resistant to irradiation (Hall 1994; Johansson et al. 2002).

The use of chemotherapy has previously been controversial, due to the fact that the efficacy of chemotherapy has been limited. The most common type of brain tumours, high-grade gliomas, tends to be extremely resistant to chemotherapy, and long-term tumour control is rarely achieved (Abe et al. 1998). Chemotherapy, however, prolongs survival for some types of brain tumours, such as oligodendrogliomas and primitive neuroectodermal tumours (medulloblastomas). Recently encouraging results have been shown after treatment with concomitant temozolomide, an alkylating agent that penetrates the blood-brain barrier, and radiotherapy. The two years survival increased from 8% in the group of patients receiving post-operative radiotherapy alone, to 29% in the group of patients treated with radiotherapy plus temozolomide (Stupp et al. 2005).

MENINGIOMAS

Epidemiology

Meningiomas are the most common benign intracranial tumours. They constitute approximately 20% of all primary intracranial tumours, with an approximately annual incidence of 6 per 100,000 (Sankila et al. 1992; Lantos et al. 1996; Louis et al. 2000). Meningiomas are most common in middle-aged and elderly patients, with a higher frequency in women. For non-resectable meningiomas, as well as for the malignant ones, the outcome can be fatal and new treatment strategies are needed (Chang & Horoupian; 1994; Bergenheim & Henriksson, 1998).

Etiology

Meningiomas, regardless of gender, seem to acquire a variety of hormone receptors during tumour genesis, and the best established is the progesterone receptor. The association between hormone receptor expression and meningiomas have been used to explain the discordant prevalence of meningiomas in females, where the overall ratio is 2:1 in the brain and up to 10:1 in the spine (Carroll et al. 1993; Black et al. 1997). The strong association with breast cancer (Rubinstein et al. 1989; Markopoulos et al. 1998) has furthermore, led to strong interest in the role of sex hormones and their receptors in etiology and progression. Moreover, an association between colorectal cancer and meningiomas in females has recently also been found (Malmer et al. 2000).

Classification

The majority of meningiomas (80-90%) are benign and classified as WHO grade I. A variety of histopathological subtypes fall into this category, including meningothelial, fibrous, and transitional meningiomas as the most common variants. Less common subtypes are psammomatous, angiomatous, microcystic, secretory, lymphoplasmacyte-rich, and metaplastic meningiomas. Between 5 and 15% of meningiomas are classified as atypical, corresponding to WHO grade II. Anaplastic (malignant) meningioma (grade III) is rare and account for 1-3% of all meningiomas (Perry et al. 1997; Louis et al. 2000). Meningiomas grouped by likelihood of recurrence and grade are summarised in Table 2.

Table 2. Meningiomas grouped by likelihood of recurrence and grade.

Low risk of recurrence and aggressive growth	WHO grade	Greater likelihood of recurrence and/or aggressive behaviour	WHO grade
Meningothelial meningioma	I	Atypical meningioma	II
Fibrous (fibroblastic) meningioma	I	Clear cell meningioma (intracranial)	II
Transitional (mixed) meningioma	I	Chordoid meningioma	II
Psammomatous meningioma	I		
Angiomatous meningioma	I		
Microcystic meningioma	I	Rhabdoid meningioma	III
Lymphoplasmacyte-rich meningioma	I	Papillary meningioma	III
Metaplastic meningioma	I	Anaplastic (malignant) meningioma	III

Modified from Louis et al. 2000.

Biology

Meningiomas are histologicallly benign tumours of the intracranial and intraspinal compartments arising from meningothelial cells of the arachnoidal layer surrounding the central nervous system (Akeysson et al. 1996). Infiltration of the tumour into the dura and overlaying bone and subcutaneous soft tissue can occur, but invasion into the neural parenchyma is usually not seen except with malignant transformation, which is very rare. However, the location in which these tumours arise has a critical impact on prognosis. The invasiveness of meningiomas is characterised by irregular groups of tumour cells infiltrating the adjacent cerebral parenchyma and may occur in histologically benign, atypical or anaplastic meningiomas. Anaplastic meningiomas are associated with a less favourable clinical outcome, since they have a higher rate of recurrence and aggressive behaviour. Some genomic alterations are associated with the formation of benign meningiomas and the progression towards atypia and anaplasia (Figure 2).

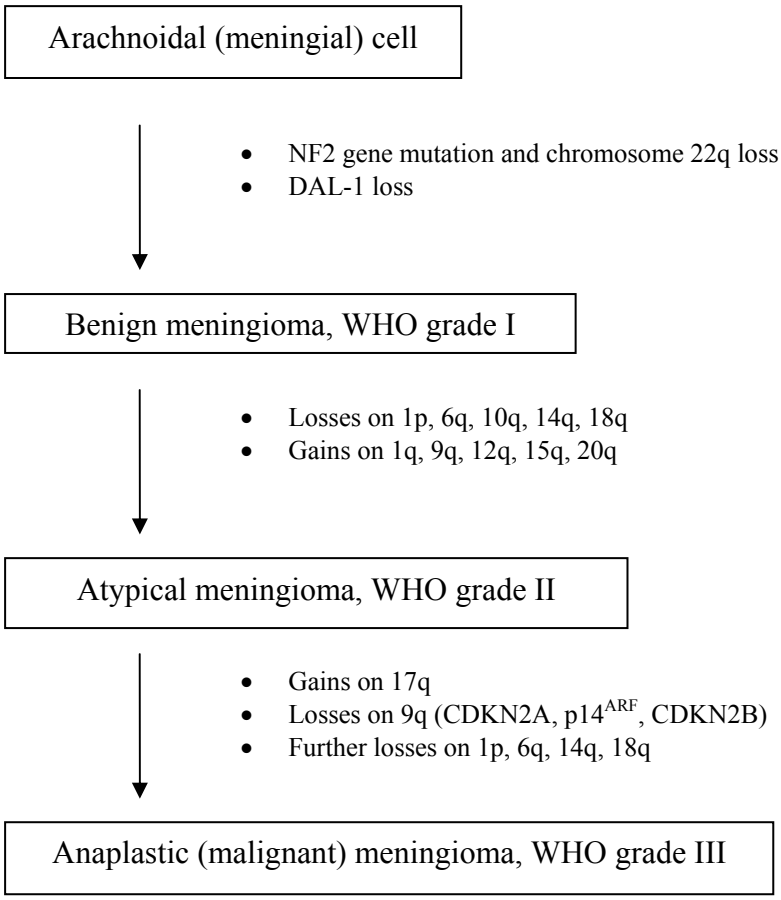

Figure 2. Hypothetic model of genomic alterations associated with the formation of benign meningiomas and progression to towards atypia and anaplasia. Modified from Lamszus K. 2004.

Treatment

Surgery is the basis of treatment for all types of meningiomas. Although some retrospective studies have shown that radiotherapy after recurrence or subtotal resection is beneficial for patients with benign meningioma (Barbaro et al. 1987, Condra et al. 1997), there is still a debate regarding the timing of radiotherapy, whether it should be given post-operatively or at the time of progression. Radiotherapy is also recommended for patients with aggressive and malignant meningiomas (Milosevic et al. 1996).

Treatments utilizing various chemotherapeutic agents have occasionally been given for patients with recurrent, unresectable, and previously irradiated meningiomas (LeMay et al. 1989). However, there are only a few reports suggesting that chemotherapy may have a role, although limited, in these tumours (Bernstein et al. 1994; Stewart et al. 1995).

MECHANISMS OF RESISTANCE

There are two general types of resistance to anticancer drugs; those that impair delivery of drugs into tumour cells and those that affect drug sensitivity. Impaired drug delivery can result from poor absorption of orally administrated drugs, increased drug metabolism or increased excretion, resulting in lower levels of drugs in the blood and reduced diffusion of drugs from the blood into the tumour mass (Bergenheim et al. 1998; Pluen et al. 2001; Jain 2001).

Failure of chemotherapy in the treatment of brain neoplasms has been mainly attributed to tumour-cell resistance. During the past decade, analysis of drug resistance of brain tumours has become a topic of much interest, and there has been some progress in the understanding of the molecular mechanisms by which brain tumour cells in general have a drug resistant phenotype. To date, most studies on drug resistance in brain tumours have focused on gliomas in view of their frequency.

The blood-brain barrier

Resistance to chemotherapy in intracerebral tumours most likely involves failure to reach therapeutic concentrations of chemotherapeutic agents in the CNS. One of the mechanisms behind this resistance is the blood brain barrier (BBB). This barrier is essential for the maintenance and regulation of the neural environment,

protecting the neural tissue from toxins, buffer variations in blood composition, and maintenance of the barrier function between blood and brain.

The cells responsible for the establishment of the BBB are the capillary endothelial cells. Compounds entering from the blood have to be transported transcellularly across the brain endothelial cells, and because of the physical nature of the BBB, transport across these barriers is heavily dependent on the lipophilicity of the compound (Jolliet-Riant et al. 1999).

The main structures responsible for the barrier properties are the tight junctions (Kniesel & Wolburg, 2000). Tight junctions seal adjacent epithelial cells in a narrow band just beneath their apical surface, and prevent the passage of molecules and ions through the space between cells. In the last ten years, the knowledge of the molecular composition of the tight junctions has markedly improved (Balda & Matter 2000). The end feet of astrocytes form a net of fine lamellae closely apposed to the outer surface of the endothelium, suggesting that inductive influences from astrocytic glia could be responsible for the development of the specialised BBB phenotype of the brain endothelium (Janzer & Raff 1997; Kacem et al. 1998). Certain drugs do cross the endothelial barrier via free diffusion thereby undergoing influx from the blood to the brain compartment. However, this influx can be immediately followed by an active efflux from brain back to blood if the drug is a substrate for any of the different active efflux transporters, such as P-glycoprotein, (Pgp) (Tsuji & Tamai 1999) which is expressed within the brain microvasculature.

In glioblastomas the BBB is disrupted resulting in cerebral oedema (Roberts et al. 2000). In contrast, this barrier is usually preserved in low-grade gliomas. The mechanisms underlying the breakdown of BBB are essentially unknown. Since non-neoplastic astrocytes are required to induce BBB features of cerebral

endothelial cells, it is conceivable that malignant astrocytes have lost this ability due to dedifferentiation. Alternatively, glioma cells might actively degrade previously intact tight junctions of the barrier. However, there is substantial controversy regarding the role of the BBB in failure to chemotherapy of intracerebral tumours (Stewart 1994).

Classical multidrug resistance

Multidrug resistance (MDR) is defined as the ability of cancer cells to become simultaneously cross-resistant to several structurally and mechanistically unrealated drugs (Endicott & Ling, 1989). Drug resistance may be present before chemotherapy (intrinsic resistance), resulting in initial treatment failure, or it can develop during chemotherapy (aquired resistance) leading to early disease progression despite initial response.

Classical multidrug resistance include resistance to hydrofobic drugs, and generally results from expression of an ATP-dependent efflux pump, named Pgp (Juliano & Ling, 1976). Pgp is a glycosylated membrane protein of molecular mass 170 kDa with broad drug specificity. This protein is encoded by the *MDR1* gene on chromosome 7, and was the first ABC transporter described belonging to the ABC subfamily B. Pgp decrease intracellular drug accumulation by acting as an ATP-driven transmembrane drug transporter, which lowers cellular drug concentrations by a bidirectional mechanism including both decreased drug uptake and increased drug efflux (Figure 3). This membrane efflux transporter is found in normal tissues, such as hepatocytes, kidney, small intestine, colon, and adrenal glands (Fojo et al. 1987; Thiebaut et al. 1987). Pgp is also known to be expressed by the microvessels of the developing brain, and also by endothelial

cells at the blood-brain barrier (Cordon-Cardo et al. 1989; Schumacher et al. 1997).

Pgp is expressed at high levels in some cancers and has been associated with clinical drug resistance. Drugs that are affected by the function of this protein include the vinca alkoids (vinblastine, vincristine), the anthracyclines (doxorubicin, daunorubicin), and the microtubule-stabilising drug paclitaxel (Ambudkar et al. 1999). Since the early 1980s many agents have been investigated for their ability to reverse Pgp-mediated multidrug resistance. Examples include verapamil, cyclosporine A, and PSC-833. Unfortunately, these agents were found to be weak inhibitors and toxic at high doses (Chan et al. 1991; Ferry et al .1996). Currently available data suggests that a subgroup of human brain tumours show intrinsic or acquired overexpression of the *MDR1* gene, which is consistent with resistance to chemotherapy (Abe et al. 1998).

Atypical multidrug resistance

Besides the classical multidrug-resistant phenotype, there are tumours with multidrug resistance caused by different mechanisms. Overexpression of alternative ABC-transporters is one important mechanism described (Dean et al. 2001). Following the discovery of Pgp, investigations of cancer cells displaying the multidrug resistance phenotype not associated with MDR1 expression led to the discovery of the MRP subfamily.

Multidrug resistance protein-1, (MRP1) is a founding member of the this subfamily, which consists of at least nine members (Cole et al. 1992, Borst et al. 2000) and belongs to the ABC subfamily C. MRP1 is a membrane-spanning protein with a molecular mass of 190 kDa that shares 15% amino acid homology with Pgp. The *MRP1* gene, encoding this protein is located at chromosome 16.

MRP1 acts as a drug efflux pump (Figure 3) and is broadly expressed in the epithelial cells of multiple tissues including the digestive, urogenital, and respiratory tracts, in the endocrine glands, and in the hematopoietic system (Flens et al. 1996).

MRP1 expression has been demonstrated in various tumour tissues and has been implicated as a component of the multidrug resistance phenomenon in cancers of the lung, colon, breast, bladder, and prostate as well as leukaemia (Nooter et al. 1995; Kruh et al. 1995). This protein has been shown to transport gluthathione conjugates of several drugs, including alkylating agents, etoposide, and doxorubicin (Schneider et al. 1994; Morrow et al. 1998). The isoflavonoid genistein has been reported to increase the daunorubicin accumulation in several MRP1 postive cell lines, but the toxicity limits its use as a resistance modifier (Versantvoort et al. 1994). Additionally, modifiers such as verapamil, cyclosporin A, and PSC 833 are usually less effective in the reversal of MRP1 (Twentyman & Versantvoort, 1996).

There is also growing evidence that increased MRP1 expression may be involved in the formation of intrinsic or aquired drug resistance in a subset of brain tumours, and particularly gliomas (Abe et al. 1994; Gomi et al. 1997; Abe et al. 1998).

Multifactorial multidrug resistance

An important issue of multidrug resistance is that cancer cells are genetically heterogenous. Although the process that results in uncontrolled cell growth in cancer favours clonal expansion, tumour cell that are exposed to chemotherapeutic agents will be selected for their ability to survive and grow in the presence of cytotoxic drugs. Therefore, in any population of cancer cells that

are exposed to chemotherapy, more than one mechanism of multidrug resistance can be present. This phenomenon has been called multifactorial multidrug resistance.

Another protein described to be involved in this type of multidrug resistance is the lung resistance protein (LRP), encoded by a gene located at chromosome 16. This protein was initially identified in a non-small-cell lung cancer (NSCL) cell line selected for doxorubicin resistance that did not express Pgp (Scheper et al. 1993). Furthermore, screening of an expression library identified LRP as the human major vault protein (MVP) (Scheffer et al. 1995), thereby implying a role for vaults in drug resistance. Vaults are ribonucleoprotein particles found in the cytoplasm of eucaryotic cells. This protein, with a molecular mass of 110 kDa, is found in the cytoplasm and on the nuclear membrane (Figure 3). LRP is not an ABC transporter protein, although it is thought to be involved in transmembrane transport and defence against nuclear toxins, perhaps in conjunction with ABC transporters present in the various cellular membranes (Scheffer et al. 1995).

LRP is highly expressed in several epithelial tissues. In cancers derived from these tissues, a variable expression of this protein is observed, with the highest expression found in colorectal tumours (Izquierdo et al. 1996). Other tumours in which expression of LRP has been reported include melanomas, osteosarcomas, and neuroblastomas (Ramani et al. 1995; Schadendorf et al. 1995). The pyridine analog, PAK-104P has been suggested to partially reverse LRP-mediated drug resistance *in vitro* (Kitazono et al. 2001). So far, no clinical studies have been published regarding modulation of LRP-mediated resistance. Althoguh studies on the expression and involvement of LRP in brain tumours are sparse, it has

been shown that glial cells in primary and secondary glioblastomas highly express this protein (Tews et al. 2000).

Resistance due to activation of detoxifying systems

Drug resistance can also result from activation of coordinately regulated detoxyfying systems, such as DNA repair. One such mechanism is the human DNA repair protein, O^6 methylguanine-DNA methyltransferase (MGMT). This protein is encoded by the *MGMT* gene, which is located at chromosome 10 (Tano et al. 1990). MGMT, acts by removing cytotoxic alkyl adducts at the O^6 position of DNA-guanine leaving a normal guanine behind, and thereby prevents the formation of DNA strand breaks (Figure 3). Thereby, MGMT protects normal cells from exogenous carcinogens and tumour cells from chemotherapeutic agents, especially alkylating agents. Tumours are known to be heterogeneous in MGMT expression and several human tumours lack MGMT expression due to abnormal promoter methylation (Citron et al. 1991). There is evidence that MGMT may not only determine tumour response but also may act as an independent predictor of prognosis (Esteller et al. 2000). A particular pivotal mechanism of individual drug resistance stems from changes in MGMT contributing to clinical resistance to alkylating chloroethylnitrosoureas and temozolomide. At present these are the leading chemotherapeutic agents for high-grade gliomas. O^6-benzylguanine is a specific inhibitor of MGMT, but conversely, mutations in the protein can cause resistance to this modulator (Dolan et al. 1990).

The intracellular localisation and hypothetic role of the multidrug resistance markers Pgp, MRP1, LRP, and MGMT described above are summarised in Figure 3.

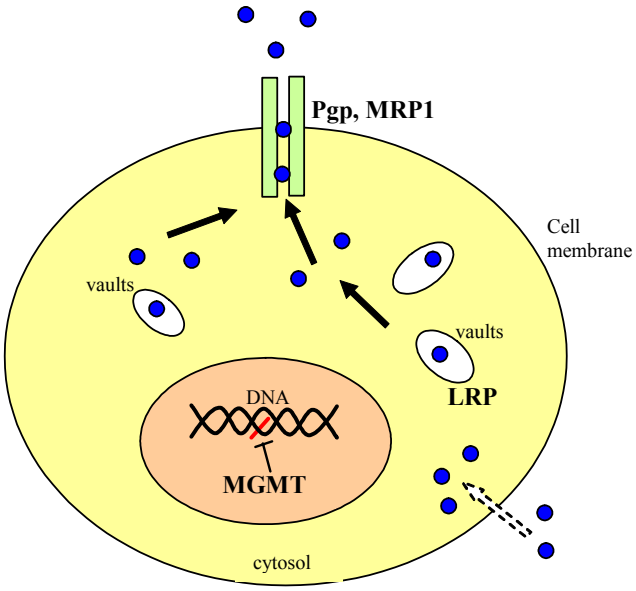

Figure 3. Simplified schematic view of the location and hypothetical role of P-glycoprotein (Pgp), multidrug resistance protein-1 (MRP1), lung resistance protein (LRP), and O^6methylguanine-DNA methyltransferase (MGMT) in cytoplasmatic and vesicular transport of drugs and/or metabolites.

Resistance mediated by reduced cellular drug uptake

Resistance can also be mediated by reduced cellular drug uptake. Water-soluble drugs or agents that enter by means of endocytosis, might fail to accumulate without evidence of increased efflux. Examples include the antifolate methotrexate, nucleotide analogues, such as 5-fluorouracil and 8-azaguanine, and cisplatin (Shen et al. 1998; Shen et al. 2000).

Resistance due to changes in apoptotic pathways

Resistance can result from defective apoptotic pathways. This might occur as a result of malignant transformation, for example in cancers with mutant or non-functional p53 (Lowe et al. 1993), or with mutated and constitutively activated EGFR (Montgomery et al. 2000).

ERBB RECEPTOR TYROSINE KINASES

The ErbB family of receptor tyrosine kinases (RTKs) couples the binding of extracellular growth factor ligands to intracellular signalling pathways regulating diverse cellular processes, including proliferation, differentiation, motility, and survival. The four closely related members of the ErbB family; epidermal growth factor receptor (EGFR, also known as ErbB1, and HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4) form homo – and/or heterodimers on binding of EGF-like or neuregulin (NRG) ligands, resulting in autophosphorylation of their cytoplasmic part (Figure 4).

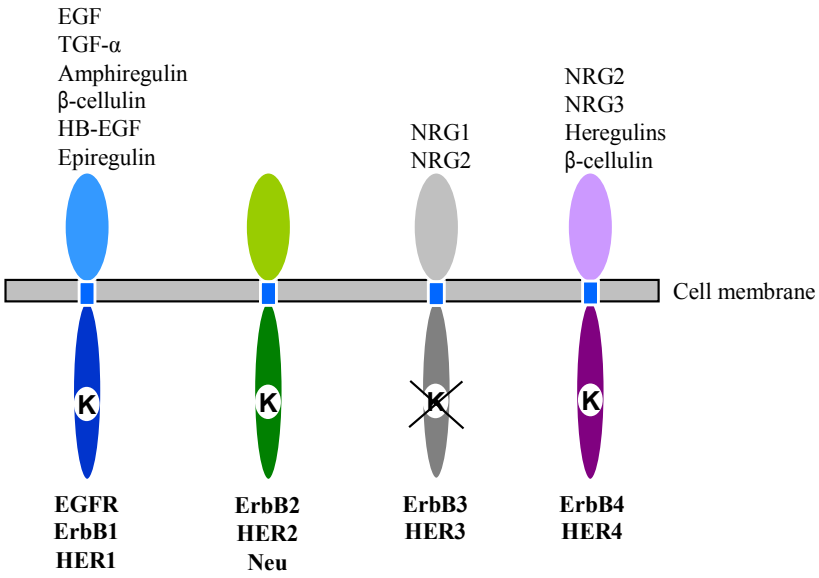

Figure 4. Schematic figure of the ErbB family members and their known ligands. K indicates the tyrosine kinase domain.

EGFR (ErbB1, HER1)

EGFR was the first human member of the ErbB family to be described (Ullrich et al. 1984). EGFR is encoded by the *EGFR* gene, which is located at chromosome 7. EGFR is a 170 kD glycoprotein that consists of an extracellular receptor domain, a transmembrane region, and an intracellular domain with tyrosine kinase function. EGFR signalling plays an important role in directing the behaviour of epithelial cells. Overexpression, gene amplification, or mutations of *EGFR* are found in multiple human tumours, including cancers of the breast, head and neck, lung, and in brain tumours of glial origin (Rasheed et al. 1999). Amplification of *EGFR* is found in approximately 50% of high-grade gliomas, suggesting that EGFR overexpression and/or gene alteration is a

late event in tumour genesis of gliomas, and is frequently observed in primary or de novo glioblastomas (Schlegel et al. 1994) (Figure 1). Amplification of EGFR correlates with a shorter survival for glioma patients receiving adjuvant therapies (Etienne et al. 1998; Hiesiger et al. 1993). Overexpression of EGFR has also been implicated in resistance to radiotherapy (Sartor et al. 2000). In addition to wild-type EGFR, cancer cells have also been shown to express various mutated EGFR molecules. The most common variant is EGFRvIII, in which part of the extracellular domain is deleted which results in constitutive and ligand-independent signalling. EGFRvIII is common in glioblastomas, breast, and ovarian tumours (Moscatello et al. 1995), but its prognostic significance is not established.

ErbB2 (HER2, Neu)

ErbB2 is encoded by the *ERBB2* gene which is located at chromosome 17 (Schechter et al. 1984). ErbB2 does not bind directly to any known ligand but functions as a co-receptor for the other members of the ErbB family. Thereby it enhances kinase-mediated activation of downstream signalling pathways (Klapper et al. 1999). ErbB2 is overexpressed in breast, cervix, colon, endometrial, esophageal, lung, and pancreatic cancers (Slamon et al. 1987; McCann et al. 1990; Weiner et al. 1990). In breast and ovarian cancer, overexpression of ErbB2 correlates with poor prognosis (Slamon et al. 1987; Meden et al. 1997). ErbB2 has been shown to be expressed both in meningiomas and gliomas, but the clinical significance is not yet established (Schwechheimer et al. 1994; Hwang et al. 1998).

ErbB3 (HER3)

ErbB3 is encoded by the *ERBB3* gene located at chromosome 12 (Plowman et al. 1990). Although ErbB3 has a tyrosine kinase domain that is highly

homologous to those of the other family members, it lacks kinase activity (Guy et al. 1994; Sierke et al. 1997). Therefore, heterodimerisation with the other three family members are crucial for cell signalling by the ErbB3 receptor. ErbB3 is overexpressed in breast, colon, prostate, ovarian, and stomach malignancies (Simpson et al. 1995; Blume-Jensen et al. 2001). In brain tumours, studies on the expression of ErbB3 are sparse, and the role of ErbB3 within these tumours is not fully elucidated (Schlegel et al. 1994).

ErbB4 (HER4)

The ErbB4 receptor is encoded by the *ERBB4* gene located at chromosome 2 (Zimonjic et al. 1995). ErbB4 has at least four isoforms with two variations in the extracellular region and two variants in the cytoplasmic region (Junttila et al. 2000). The ErbB4 receptor has been proposed to act as a suppressor of malignant transformation. ErbB4 expression is associated with increased survival for patients with ErbB2 positive tumours (Suo et al. 2002). In addition, ErbB4 is strongly down-regulated in renal (Thomasson et al. 2004), prostate (Lyne et al. 1997), and pancreatic cancer (Graber et al. 1999) compared to the corresponding normal tissues. However, in childhood medulloblastomas co-expression of ErbB4 and ErbB2 correlates with shorter survival (Gilbertsson et al. 1997).

ErbB family signalling pathways

The cellular outcome of activation of the ErbB family is cell context dependent and depends upon the signalling pathways induced, which in turn are determined by the composition of the receptor dimers and the identity of the ligand. The ErbB network is involved in many human cancers, and dysregulation of the many signalling pathways induced through the ErbB receptors can promote

many different properties of neoplastic cells, such as proliferation, migration, angiogenesis, stromal invasion and resistance to apoptosis. ErbB family induced signalling pathways include the Ras/Raf/MAPK pathway, the PI3-K/Akt pathway, and the PLC-γ pathway (Figure 5) (Yarden et al. 2001).

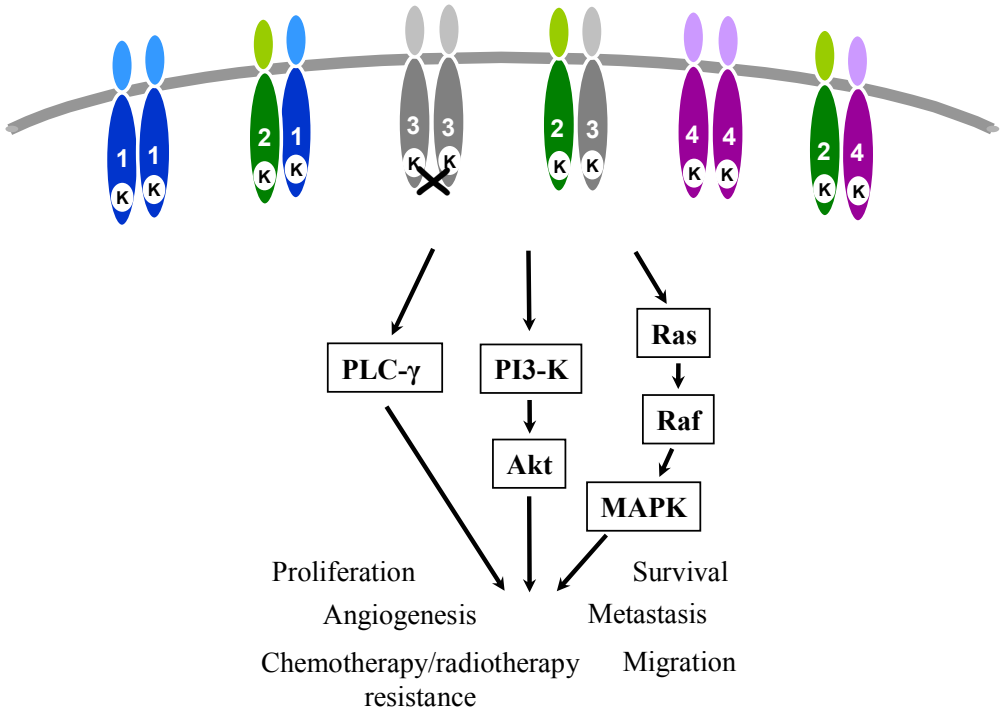

Figure 5. Schematic view of three major ErbB family signalling pathways.

REGULATION OF ERBB FAMILY PATHWAYS

Endogenous inhibitory pathways

Endogenous inhibitory pathways are essential for terminating EGFR activity (Sweeney et al. 2004). Therefore it is of interest to further investigate these

negative ErbB receptor pathways and the mechanism by which they are overcome by tumour cells. Furthermore, whether these pathways may be utilized in the clinical treatment of patients is largely unexplored. Recently, the leucine-rich repeats and immunoglobulin-like domains (LRIG) family was identified (Suzuki et al. 1996; Nilsson et al. 2001; Holmlund et al. 2004: Guo et al. 2004). LRIG1 down-regulates the ErbB family receptor tyrosine kinases by enhancing receptor ubiquitylation and degradation (Gur et al. 2004; Laederich et al. 2004), which suggest that LRIG1 might act as a tumour suppressor by antagonizing growth factor signalling (Hedman et al. 2002; Thomasson et al. 2003). Whether this molecular function is shared by the other members of the LRIG family is not known.

Therapeutic inhibition of ErbB signalling pathways

Many different strategies to interfere with ErbB family-mediated signalling are being investigated and will hopefully translate into safe and effective treatments.

Several monoclonal antibodies have been developed that target different members of the ErbB family. Cetuximab (IMC-225) is an antibody directed against EGFR, which recently has received approval for the treatment of colorectal cancer (Herbst et al. 2002). This antibody binds to the extracellular domain of EGFR, thereby preventing tyrosine kinase activation, inhibiting cell growth, and in some cases induces apoptosis. Trastuzumab is an antibody directed against the extracellular domain of ErbB2. This antibody is approved for use in breast cancers with overexpression of ErbB2.

Another approach to interfere with ErbB family signalling is the use of small molecular RTK inhibitors. In contrast to the monoclonal antibodies, this class of

agents does not down-regulate EGFR receptor expression. EGFR inhibitors approved for clinical trials are gefitinib (ZD1839) and erlotinib (OCI-774).

AIMS OF THE PRESENT STUDY

The principal purpose of this thesis is to increase the understanding regarding multidrug resistance and the ErbB family in brain tumours.

The specific aims were:

- To investigate the importance of multidrug resistance in low- and high-grade gliomas and meningiomas by analysing the expression of P-glycoprotein (Pgp), Multidrug resistance protein-1 (MRP1), Lung resistance protein (LRP), and O^6methylguanine-DNA methyltransferase (MGMT) in primary brain tumours, and their relation to clinical data.

- To evaluate the expression and clinical importance of the ErbB family members (EGFR, ErbB2- 4) in gliomas and meningiomas.

- To explore the effects of irradiation on the *in vitro* and *in vivo* expression and functional activity of Pgp in glioma cells and endothelial cells.

- To evaluate if the cytotoxic effects of irradiation are influenced by various expression in EGFR and ErbB2, and whether timing of administration of the tyrosine kinase inhibitor ZD1839 is of importance in order to achieve an optimal treatment effect.

MATERIAL AND METHODS

The methods used are summarised below. For further details about the methods, see the papers referred to by their roman numerals.

Cell lines (Papers I, III, IV)

The human glioma cell lines (U-251MG, U-118MG, U-138MG, U-343MG, U-105 MG, SF-767), all characterised as glioblastomas, the rat glioma cell line (BT4C) characterised as a glial tumour with histopathological appearance of a gliosarcoma (Laerum et al. 1977; Bergenheim et al. 1994), and the immortalized rat brain vascular endothelial cell line (RBE4) (Regina et al. 1998) were used for the *in vitro* part of this thesis.

The human and rat glioma cell lines, were all cultured in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% fetal calf serum and 50 µg/ml gentamycin, with one exception for the *in vitro* studies on the U-251MG cell line in Paper I, which was cultured in Eagle's MEM supplemented with 10% fetal calf serum and 50 µg/ml gentamycin. The rat brain endothelial cell line, grown on rat-tail collagen I-coated surface, was maintained in Ham's F-10 supplemented with 1 µg/L bFGF. All cell lines were cultured at 37° C with 5% CO_2 for at least three days until the cell growth was exponential, and medium was changed every third day. The cells were harvested and treated in appropriate way for the different analyses.

Rat glioma model and tissue sampling (Paper I)

In this study, a model based on a transplacental nitrosourea induced tumour was used. This tumour has previously been demonstrated to have common features with human anaplastic gliomas (Learum et al. 1977; Bergenheim et al. 1994). The BT4C glioma cells, derived from this tumour, were grown as monolayers

for one week before implantation. The cells were trypsinised and diluted in DMEM supplemented with 5% BDIX rat serum to 20,000 cells/5 μL.

Inbred rats were anaesthetised by i.p. administration of 1.8 mg/kg of a 1:1 mixture of Hypnorm® (fluanisonum 10 mg/mL, and fentanylum 0.2 mg/mL) and Dormicum® (midazolam 5 mg/mL).

Twenty thousand cells were transplanted under stereotactic conditions in the caudate nucleus allowing at least 5 min for injection and withdrawal of the needle to prevent cellular reflux and extracerebral spread of tumour cells. The drill hole was closed with bone wax. To ensure cell viability, the cell suspension was kept on ice during the implantation procedure and cells were stained with trypan-blue.

Seven or fourteen days after irradiation, three animals from each group were perfusion fixated with 4% paraformaldehyde before they were sacrificed. The brain tissues were immediately frozen in liquid-nitrogen and stored in -80°C, until post-fixation in 70 % ethanol and embedding in paraffin was performed.

Radiotherapy (Papers I, IV)

Irradiation of the human (251MG, SF-767), rat (BT4C) glioma cell lines, and the rat brain endothelial cell line (RBE4) was delivered in single doses of 2, 8 Gy (PaperI) or 2, 4, 6 Gy (Paper IV). Treatment was given with 195 kV x-rays at 22°C using a 0.5 mm Cu filter. The dose rate was 1 Gy/min at the level of the irradiated cells and the source-phantom distance was 500 mm (Hendersson et al. 1981; Bergenheim et al. 1995). The culture plates, or culture bottles, with the cell cultures were placed on a 150 mm thick lucite block in order to allow full back scatter. After irradiation of the cell lines the medium was changed and the cells were cultured for varying length of time before they were analysed.

Irradiation of the rats was given as whole brain treatment using a conventional 4 MV linear accelerator. A single dose of 2 or 8 Gy was given 10 days after tumour implantation. Irradiation was performed on conscious rats temporarily immobilized in a net restrained and the body covered by a lead protection. The doses and technique were chosen according to previous experience with the purpose to obtain a moderate tumour effect without inflicting serious normal brain tissue damage (Bergenheim et al. 1995; Johansson et al. 1999). Source surface distance was 0.66 m and the dose rate 2 Gy/min.

Drug treatment (Paper IV)

The selective oral epidermal growth factor receptor-tyrosine kinase inhibitor, ZD1839 (Iressa, gefitinib) (kindly provided from Astra Zeneca, Aldery Park, UK), was used for the *in vitro* experiments. ZD1839 was dissolved in 100% DMSO to a stock solution of 1 g/L, and further diluted in culture media to concentrations ranging from 1-10 mg/L. The cell lines were treated with ZD1839 for different times of exposure.

RT-PCR (Paper I)

Total RNA was isolated from tissues and the irradiated glioma cell lines using TRIzol Reagent according to the manufacturer instructions. RT-PCR was performed using 1 μg total RNA and 1st Strand cDNA Synthesis kit. cDNA synthesis was followed by a PCR protocol. Forward and reverse primers designed to amplify *MDR1* (human), and *mdr1a*, *mdr1b* (rat) genes (Regina et al. 1998) were used for the RT-PCR reactions.

Quantitative realtime RT-PCR (Paper III)

RNA samples were run in triplicate using 100 ng of total RNA from cell lines, 7.5 pmoles forward and reverse primers, 5 pmoles probe, and 20 U RNase out

per reaction. Relative quantification was performed by comparing the threshold cycle values (C_t) of the samples with standard curves generated using cloned cDNAs of respective genes. To correct for differences in RNA quality and quantity, the apparent levels of 18S rRNA in respective samples were used to normalise the levels of the different ErbB family members.

Calcein accumulation assay (Paper I)

The calcein assay is a functional probe for measurement of Pgp transport activity (Holló et al. 1994, Liminga et al. 1994; Jonsson et al. 1999) and other types of multidrug resistance drug efflux pumps such as MRP1 (Homolya et al. 1993; Holló et al. 1998). The acetoxymethyl ester (AM) derivative of calcein is a hydrophobic fluorescein derivative that is actively extruded by drug efflux transporters. Calcein (AM) is highly soluble and penetrates plasma cell membranes very fast. It is practically non-fluorescent, but becomes fluorescent and hydrophilic after cleavage of the ester bond by intracellular esterases, and is thereafter no longer a substrate of the drug efflux transporters. Cells expressing drug efflux transporters activity rapidly remove the non-fluorescent probe calcein (AM), resulting in decreased accumulation of the fluorescent dye calcein in the cytoplasm department.

The cells were harvested and plated in microtiter plates, and incubated at 37°C for 24 h with culture media only. The cells were washed twice with PBS containing 5mmol/L glucose, and calcein (AM) with or without the Pgp modulator verapamil was then added to the media, and the cells were further incubated for 0–120 min. The fluorescence with excitation at 495 nm and emission at 515 nm was measured on a Perkin Elmer LS50B luminescence fluorometer.

Immunohistochemistry (Paper I)

To confirm the expression of Pgp in the cell lines, immunohistochemical evaluation was performed on cytospin preparations. For antigen retrieval, slides were immersed in citrate buffer. Endogenous peroxidase was blocked, followed by non-immune serum blocking. A monoclonal antibody, recognizing an intracellular epitope of Pgp, was applied as primary antibody. Biotinylated secondary antibody was added, subsequently, streptavidin-biotin horseradish peroxidase enzyme conjugate was added. Finally, the staining reaction was developed in 3,3´-diaminobenzidine, and cytospin preparations were counterstained with Mayer's haematoxylin. For negative controls, the primary antibody was omitted in the process of immunostaining. The staining of the cultured cells was semi-quantitatively evaluated.

Immunohistochemical evaluation of *in vivo* expression of Pgp in the rat glioma model was performed on paraffin embedded tissue specimens, using a double-staining technique with two different antibodies. A monoclonal antibody against Pgp, was applied as primary antibody. Biotinylated secondary antibody was added, followed by incubation in enzyme conjugate, ABC-AP. The staining reaction was developed by addition of AP-substrate, together with levamisole to block endogenous alkaline phosphates. After rinsing the sections in ethanol and distilled water staining with the second primary antibody was performed. The sections were incubated in DS enhancer and blocking in non-immune serum was performed.

Polyclonal antibody, reacting with von Willebrand Factor present in endothelial cells was applied. Biotinylated secondary antibody was added and endogenous peroxidase was blocked. Streptavidin-biotin horseradish peroxidase enzyme conjugate was subsequently added. Finally, the staining reaction was developed

in 3,3´-diaminobenzidine, and sections were counterstained with Mayer's haematoxylin. For negative controls the primary antibodies were omitted in the process of immunostaining. The immunohistochemical staining was evaluated by estimating the number of positive cells semi-quantitatively.

Immunoblot analysis (Paper IV)

Protein lysates from cell lines were separated by electrophoresis on TRIS-acetate NuPAGE gels, and transferred to PVDF membranes using an Xcell II Mini-Gel blot module. Specific proteins of interest were detected by incubation with suitable antibodies and the blots were visualised by enhanced chemiluminescence technique, ECL (Amersham Biosciences, Sweden). The primary antibodies used were against EGFR, phosphorylated EGFR, ErbB2, Akt, phosphorylated Akt, and actin.

Quantification of apoptosis by TUNEL technique (Paper IV)

TUNEL (TdT-mediated dUTP nick end labelling) technology detecting nuclear DNA fragmentation was used for quantification of apoptosis. The free 3´-OH terminal was labelled with modified fluorescence-labelled nucleotides (dUTP) by catalysis of TdT (terminal deoxynucletidyl transferace). Cells were harvested with trypsin and then diluted to 2 x 10^7 cells/mL in 100 μL cell suspensions. The cells were fixed in 2% paraformaldehyde, and then permeabilized with 0.1% triton x-100 in 0.1% sodium citrate, followed by incubation with TUNEL read mix (Roche, Mannheim, Germany). TUNEL marked DNA fragmentation was determined with use of a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Fluorometric microculture cytotoxicity assay (FMCA) (Paper IV)

To quantify the cytotoxic effects of ZD1839 and irradiation, fluorescein diacetate (FDA) was used in a fluorometric microculture cytotoxicity assay (FMCA) (Larsson et al. 1989). FDA is membrane permeable and is cleaved to fluorescent fluorescein by intracellular esterases. The esterase activity is dependent on the cell viability and integrity of the cell membrane. The uptake of FDA only occurs in viable cells, and when FDA is cleaved to fluorescein, it will be retained intracellular. Thus, the amount of fluorescence will correlate to the number of viable cells.

Cells were harvested and plated in microtiter plates. Cells were cultured until cell growth was exponential before ZD1839 was added to the medium at appropriate concentrations, or single dose irradiation was performed. Plates were incubated at 37°C for six days, and the media was renewed with or without ZD1839 after 3 days. ZD1839 was solubilized and delivered in DMSO and control samples were treated with DMSO only. Cells were initially washed, and PBS containing 10 mg/L FDA was added to each well and plates were incubated in 37°C for 50 min, followed by fluorescence determination using 485 and 538 nm for excitation and emission, respectively.

Statistical analyses (Papers I, IV)

In paper I, results are given as mean values with standard errors of means (S.E.M). The statistical significance of difference between groups was determined by one-way analysis of variance (ANOVA). In paper IV, values are expressed as mean and standard deviation. Treatment groups were compared using the Mann-Whitney U-test. Statistical analyses were performed using SPSS for Windows version 11.5 (SPSS Inc., Chicago, IL). Curve estimation for

calculation of IC50 values was performed using quadratic regression. Statview 4.11 for the Macinotsh computer was used for regression analysis.

PATIENTS

Tumour tissue sampling (Papers II, III)

Available brain tumour samples and clinical data were obtained from patients during 1987-2001. Features of the patients are presented in Paper II (Tables I and II) and Paper III (Tables I and II). Tumours were reviewed and classified by a neuropathologist according to the World Health Organisation (WHO) classification of tumours of the central nervous system (Kleihues & Cavanee 2000).

Formalin-fixed and paraffin embedded tumour tissues from 18 astrocytomas, 16 oligodendrogliomas, and 22 meningiomas, were used for the immunohistochemical evaluation in Paper II. For the studies in Paper III, the tumour samples were extended to 27 astrocytomas, 17 oligodendrogliomas, and 26 meningiomas. Fresh frozen tumour tissue available from 17 of the astrocytomas and 9 of the meningiomas were used also for RNA extraction and real-time RT-PCR analysis. Total protein available from 7 of the astrocytomas and 5 of the meningiomas were also used for immunoblot analysis (see Figure 6).

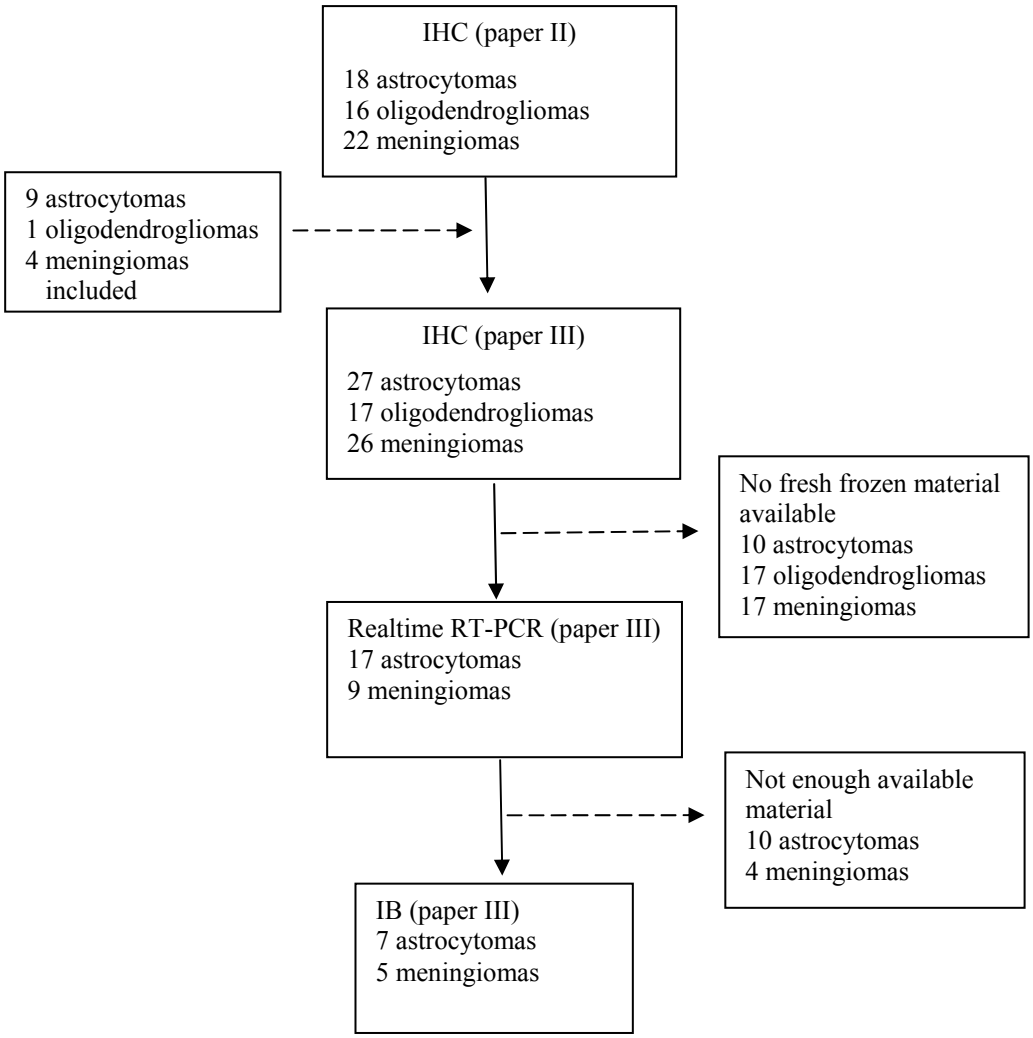

Figure 6. Schematic illustration of how the tumour tissues have been used for different analyses in papers II and III. IHC = immunohistochemistry, RT-PCR = reverse transcriptase-polymerase chain reaction, IB = immunoblot analysis.

Quantitative realtime RT-PCR (Paper III)

RNA samples were run in triplicate using 20 ng of total RNA from tumour tissue, 7.5 pmoles forward and reverse primers, 5 pmoles probe, and 20 U RNase out per reaction. Relative quantification was performed by comparing the threshold cycle values (C_t) of the samples with standard curves generated using

cloned cDNAs of respective genes. To correct for differences in RNA quality and quantity, the apparent levels of 18S rRNA in respective samples were used to normalise the levels of the different ErbB family members.

Immunohistochemistry (Papers II, III)

To confirm the expression of Pgp, MRP1, LRP, MGMT, and the ErbB family members, deparaffined sections of brain tumour tissues were immunohistochemically analysed by using specific primary antibodies. Endogenous peroxidase activity was blocked, and slides were placed in citrate buffer and pretreated in microwave oven followed by incubation with none-immune serum. The antibodies were incubated overnight at 4°C, however, antibodies against ErbB2- 4 were incubated for 1 h at room temperature. Subsequently slides were incubated with biotinylated secondary antibody, and thereafter HRP-Streptavidin. The staining reaction was developed by 3,3´- diamino-benzidine, and sections were counterstained with Mayer's haematoxylin. Samples of normal intestine tissue were used as positive staining controls. Staining with isotype-matched mouse and rabbit IgG antibodies was performed as negative controls. The staining was semi-quantitatively evaluated.

Immunoblot analysis (Paper III)

Protein lysates from tumour tissues were separated by electrophoresis on TRIS-acetate NuPAGE gels, and transferred to PVDF membranes using an Xcell II Mini-Gel blot module. Specific proteins of interest were detected by incubation with suitable antibodies and the blots were visualised by enhanced chemiluminescence technique, ECL (Amersham Biosciences, Sweden). The primary antibodies used were against EGFR, ErbB2, Erb3, and ErbB4.

Statistical analyses (Papers II, III)

Distribution of different parameters was statistically analysed by using the Pearson chi-square test. A p-value of < 0.05 was considered statistically significant. When analysing the results related to histopathological diagnosis, sex, age, and survival the numeric values were transformed and binary logistic regression was performed. For survival analysis, the immunohistochemical staining was grouped into no or $< 20\%$ immunoreactivity (low) vs. $> 20\%$ immunoreactivity (high); survival was calculated with the Kaplan-Meier method and comparison between study groups was performed with the log-rank test. All statistical analyses were performed using SPSS for Windows version 11.0 (SPSS Inc., Chicago, IL).

ETHICAL CONSIDERATIONS

The animal experiments were approved by the local ethics committee for animal research. Care was taken not to expose animals to unnecessary suffering and the number of animals was kept as low as possible.

The study of the clinical tumour samples was approved by the local ethics committee for human research.

RESULTS AND DISCUSSION

MULTIDRUG RESISTANCE IN GLIOMAS AND MENINGIOMAS (I, II)

A major obstacle in using chemotherapy is intrinsic or acquired multidrug resistance, explaining the rather modest effects of chemotherapy when treating many solid tumours. Among the factors known to be involved in these mechanisms the most studied are Pgp, MRP1, LRP, and MGMT.

Pgp and MRP1 decrease intracellular drug accumulation by acting as ATP-driven transmembrane drug transporters (Juliano & Ling, 1976; Cole et al. 1992). LRP is thought to be involved in transmembrane transport and defence against nuclear toxins (Scheffer et al. 1995). The DNA repair protein, MGMT, acts by removing cytotoxic alkyl adducts, and thereby prevents the formation of DNA strand breaks (Tano et al. 1990). Malignant gliomas and meningiomas are brain tumours with a low or no sensitivity to presently used chemotherapeutics. Although the cause to this multidrug resistance can at least partially be explained by function of specific molecules, there are still controversies regarding the real clinical importance of these markers in human brain tumours.

Pgp, MRP1, LRP and MGMT expression in gliomas (I, II)

The immunohistochemical evaluation of the expression of Pgp, MRP1, LRP, and MGMT, in formalin-fixed and paraffin embedded tumour tissues from glioma patients was evaluated, and the findings were related to clinical data (II).

A most notable observation was that, regardless of the grade of malignancy, a marked heterogeneity in the expression of the different resistance markers was evident. Expression of Pgp and MRP1 were mainly found in the capillary endothelium and in single scattered tumour cells surrounding blood vessels. In

accordance with previous studies a higher number of Pgp and MRP1 positive tumour cells were found in high-grade tumours (von Bossanyi et al. 1997; Ashmore et al. 1999). On the other hand, we found that LRP was expressed only in the tumour cells, which are in discrepancy with a study by Tews et al. 2000 that demonstrated expression of LRP both in tumour cells and blood vessels in gliomas.

In the intracranial *in vivo* rat glioma model, Pgp was expressed in the capillary endothelium but not detected in tumour cells (I), an observation which is supported by others (Regina et al. 1998). Since drug efflux mechanisms are an integrated part of the blood-brain barrier it is plausible to postulate that Pgp could be one of several factors explaining the poor response to chemotherapy in the treatment of malignant gliomas (Begley, 2004). Nevertheless, the role of the blood-brain barrier (BBB) in multidrug resistance of intracerebral tumours must be further evaluated (Stewart et al. 1994; Ashmore et al. 1999).

MGMT is a repair protein that has been suggested to be involved in drug resistance. In earlier studies expression has been found mainly in high-grade astrocytomas (Nutt et al. 2000; Nakamura et al. 2001). However, in our study a high expression of MGMT was seen both in astrocytomas and oligodendrogliomas, independent of the grade of the tumours. This observation could be of clinical importance, since the primary mechanism behind resistance to temozolomide, an alkylating agent used for treatment of glioblastomas, involves an enhanced activity of MGMT (Hegi et al. 2005). Moreover, the pronounced heterogeneity in the expression among tumour cells in the same tumour emphasises the need of a treatment approach using drugs with different mechanisms of action. Thus, these results strongly point out the need for an individualised treatment strategy, also highlighted by the indication of a shorter

survival for patients with low-grade gliomas and high expression of Pgp. A higher number of patients must, however, be evaluated before any firm conclusions can be drawn.

Pgp, MRP1, LRP and MGMT expression in meningiomas (II)

Although a distinct and prominent immunohistochemical staining of the different markers was obvious in the capillary endothelium of almost all meningiomas analysed, a heterogeneous expression of Pgp, MRP1, LRP, and MGMT was obvious. A novel finding in our study was the occurrence of Pgp and MGMT in clusters of tumour cells surrounding the capillary endothelium in transitional meningiomas. This observation is supported by an earlier study that demonstrated the occurrence of these proteins also in tumour cells, especially in atypical and malignant meningiomas (Tews et al. 2001). The variation in the expression of the analysed markers might contribute to a multidrug resistant phenotype also in subgroups of meningiomas, and thus could contribute to the known resistance to chemotherapy in these tumours.

EGFR-FAMILY IN GLIOMAS AND MENINGIOMAS (III)

Amplification and mutation of EGFR appears in more than 50% of malignant gliomas and correlate with a shorter survival (Etienne et al. 1998; Barker et al. 2001). Studies regarding ErbB2-4 and their clinical significance in gliomas and meningiomas are, on the other hand sparse.

In this study, the expression of the ErbB family members in different primary brain tumours were analysed, and the results were correlated to various parameters of clinical interest.

EGFR, ErbB2- 4 expression in gliomas (III)

Quantitative realtime RT-PCR and immunohistochemical analyses showed high mRNA and protein expression of EGFR in high-grade gliomas, whereas mRNA and protein expression of ErbB4 was most pronounced in low-grade gliomas.

ErbB4 has recently been suggested to be a tumour suppressor in breast, renal, prostate, and pancreatic cancer (Suo et al. 2002; Thomasson et al. 2004; Lyne et al. 1997; Graber et al. 1999). Thus, the high expression of ErbB4 in low-grade gliomas compared to high-grade gliomas might indicate that ErbB4 acts as a suppressor of malignant transformation also in these tumours. This observation was also mirrored by the low ErbB4 mRNA expression in the glioma cell lines analysed, all derived from glioblastomas. However, by quantitative real-time RT-PCR, ErbB4 mRNA was present at relatively high levels also in a few high-grade gliomas. This possibly reflects that brain tumour tissues are heterogeneous and contain a mixture of cells of various origins and grade of differentiation. Since only a limited amount of each brain tumour tissue were available, it is possible that tumour heterogeneity might explain the results.

A novel observation in low-grade gliomas was a significant shorter overall survival for patients with high EGFR protein expression. This finding, together with our previous observations that Pgp is highly expressed in low-grade gliomas (Paper II), might indicate the existence of a more malignant subtype of low-grade gliomas. In this context, it is of interest to emphasise the results that ^{11}C-methionine PET scan could identify low-grade gliomas with a more aggressive behaviour (Ribom et al. 2001). If this observation is confirmed in a larger study, high EGFR protein expression in tumours characterised today as low-grade gliomas could constitute a marker for a phenotype that might benefit from specific post-operative treatment.

Heterogeneity in the expression of the different ErbB family members shown by this study further stresses the clinical importance of an individualised management of patients with brain tumours. The results also suggest that tumour grading systems currently used might be improved by using additional biomarkers as discussed by others **(**Dolittle, 2004; Bäcklund, 2005).

EGFR, ErbB2- 4 expression in meningiomas (III)

In the majority of the meningiomas analysed, expression of EGFR, ErbB2, and ErbB4 mRNA was detected by quantitative realtime RT-PCR. Immunohistochemical analyses of the meningiomas showed high ErbB2 protein expression located mainly to the capillary endothelium, indicating that this protein is involved in tumour angiogenesis even in these tumours.

The meningiomas analysed in this study were all of WHO-grade I, and in several of these tumours high expression of ErbB2 mRNA and protein were detected. This is in concordance with a previous study, which reported decreased ErbB2 expression with increasing histopathological grade of meningiomas (Chozick et al. 1996). Whether these changes play a direct role in the pathogenesis of meningiomas or merely is a consequence of malignant transformation needs to be further investigated.

IRRADIATION AND MULTIDRUG RESISTANCE (I)

Patients with malignant glioma are usually treated with radiotherapy followed by chemotherapy. Data on whether radiotherapy affects the multidrug resistance (MDR) in gliomas are sparse. A previous study has demonstrated that radiotherapy significantly induce expression of Pgp in primary oral cancer (Ng et al. 1998).

The influence of irradiation on the expression of Pgp, as well as the functional activity of drug efflux pumps in cultured human glioma cells (251MG), rat (BT4C) glioma cells, and immortalized rat brain vascular endothelial cells (RBE4) was evaluated.

An increased and retained functional calcein efflux activity in brain vascular endothelial cells but not in glioma cells was demonstrated following irradiation. The calcein assay is a functional probe for measurement of Pgp transport activity (Holló et al. 1994), and other types of multidrug resistance drug efflux pumps such as MRP1 (Holló et al. 1998). The increased calcein efflux activity of the rat brain vascular endothelial cell line was obvious already after two hours, and was retained for at least fourteen days after irradiation. Verapamil, a known inhibitor of Pgp **(**Arboix et al. 1997**)**, hampered most of the calcein efflux activity of the rat brain vascular endothelial cell line, suggesting that the activity is at least partially Pgp-mediated.

The results in this study indicate that Pgp expressed in the endothelium contribute to multidrug resistance in human gliomas *in vivo*, since calcein efflux was activated only in the endothelial cells. Thus, the occurrence of multidrug resistance in malignant gliomas is probably highly dependent on endothelial cells in blood-brain-tumour barrier. It is also a possibility that the poor efficacy of chemotherapy following radiotherapy is explained by radiation-induced activity of Pgp. In order to enhance the efficacy of chemotherapy in combination with radiotherapy, it may be essential to inhibit drug resistance pump activity of endothelial cells, or deliver chemotherapy prior to radiotherapy.

IRRADIATION AND INHIBITION OF EGFR SIGNALLING (IV)

Few data on the effects of the selective EGFR tyrosine kinase inhibitor ZD1839 (gefitinib) on glioma growth have been published, especially when this agent is combined with radiotherapy (Li et al. 2003; Sundberg et al. 2003; Learn et al. 2004). Therefore, the effects of different schedules for administration of ZD1839 and irradiation were evaluated using two human glioma cell lines (251MG, SF-767), a rat glioma cell line (BT4C), and an immortalized rat brain endothelial cell line (RBE4). These cell lines express EGFR and ErbB2 to a varying degree.

The results showed that ZD1839 had different effect on the radiation-induced cytotoxicity in the analysed cell lines. Treatment with ZD1839 during 30 minutes prior to irradiation, as well as post-irradiation treatment with ZD1839 increased the cytotoxicity in all cell lines except for the human glioma cell line 251MG. In this cell line, a most striking observation was an antagonistic effect of short pre-treatment with ZD1839. On the other hand, pre-treatment for 24h prior to irradiation increased the cytotoxicity only in the human glioma cell line SF-767 and in the rat glioma cell line BT4C. Moreover, there was no strong correlation between the levels of EGFR and the response to ZD1839, which also have been seen in non-small cell lung cancer (Bailey et al. 2003; Cortes-Funes et al. 2003).

The rat brain endothelial cell line had no EGFR expression, and in the rat glioma cell line the EGFR expression was low, however, both cell lines expressed ErbB2. These cell lines were sensitive to ZD1839 alone and when combined with irradiation. Similar results have been reported for human breast cancer cell lines, suggesting that the clinical efficacy of ZD1839 should be tested also in patients with ErbB2 overexpressing tumours (Moasser et al. 2001).

Since different treatment schedules resulted in varying response in the analysed cell lines, duration of incubation with ZD1839 as well as the time for administration of ZD1839 in relation to irradiation, seems to be important for the cytotoxicity of irradiation. These results indicate that more or less inaccurate timing might hamper positive effects when selective inhibitors of signal transduction are combined with conventional therapies. Our results are in accordance with an *in vitro* study indicating that the concentration and schedule of ZD1839 administration in combination with irradiation is crucial in order to obtain radio-sensitizing effects (Stea et al. 2003).

Stimulation with EGF induced phosphorylation of EGFR and Akt in the human glioma cell lines 251MG and SF-767 only. EGFR phosphorylation was inhibited by ZD1839 in both human glioma cell lines. However, ZD1839 failed to inhibit Akt phosphorylation in the 251MG cells, which might explain why ZD1839 had different cytotoxic response in this glioma cell line as discussed above. This observation finds support in preclinical studies suggesting that activation of Erk and Akt are down-regulated by ZD1839 (Janmaat et al. 2003; Campiglio et al. 2004) and that persistent activation of the apoptotic pathways has a role in resistance to ZD1839 (Magne et al. 2002; Janmaat et al. 2003).

The increased cytotoxicity seen as a result of the different treatment schedules involved increased nuclear DNA fragmentation in the cells. This might indicate that timing of ZD1839 treatment in relation to irradiation also seems to be of importance for induction of apoptosis in the analysed cell lines. However, in the human glioma cell line 251MG, treatment with ZD1839 revealed somewhat conflicting results regarding the cell viability, in relation to nuclear DNA fragmentation. When the 251MG cell line was analysed six days after irradiation, treatment with ZD1839 during 30 minutes prior to irradiation

protected the cells from the effects of irradiation. However, an increased nuclear DNA fragmentation was obvious after the same treatment. In order to clarify these conflicting results, this cell line was also analysed three days after irradiation. At that time, increased cell viability was correlated to no increased nuclear DNA fragmentation in the cells. Furthermore, *in vitro* cell proliferation measured by cell numbers after the same treatment, verified these results of the FMCA assay.

A most pronounced effect on nuclear DNA fragmentation was observed in the rat brain endothelial cell line (RBE4), which is in accordance with a study suggesting that inhibition of EGFR leads to apoptosis of endothelial cells and reduction in neovascularity (Bruns et al. 2000). Thus, endothelial cells may represent principal targets for irradiation, and tumour cell death may represent a secondary event (Denekamp, 1982).

These results warrant further investigations regarding the ability of ZD1839 and similar drugs to modify the irradiation response of endothelial cells, especially when considered that malignant gliomas are highly vascularised tumours.

CONCLUSIONS

- A pronounced heterogeneity in protein expression of the multidrug resistance markers Pgp, MRP1, LRP, and MGMT was evident in both low -and high-grade gliomas and meningiomas. There was a tendency for a shorter survival in patients with low-grade gliomas and high expression of Pgp. In the *in vivo* rat BT4C glioma model, Pgp was expressed in the vascular endothelium of tumour tissue and surrounding normal brain, but not in tumour cells.

- Irradiation increased the functional activity of drug efflux pump mechanisms in rat brain vascular endothelial cells, but not in glioma cells *in vitro*. However, the expression of the human *MDR1*, rat *mdr1a*, *mdr1b* genes and Pgp in human and rat glioma cells were not affected by irradiation.

- A heterogeneous expression of the ErbB family members was found in gliomas and meningiomas. EGFR was usually higher expressed in high-grade gliomas, while ErbB4 was higher expressed in low-grade gliomas. Patients with low-grade glioma and high EGFR protein expression had a significantly shorter overall survival, compared to patients with no or low EGFR protein expression.

- The timing for administration of ZD1839 in relation to irradiation is important in order to obtain optimal cytotoxic effects in glioma cells and endothelial cells. There was a large variation in the effects of ZD1839 between different cell lines when combined with irradiation, with even signs of an antagonistic effect. No clear correlation between EGFR expression and response to ZD1839 was found in the analysed cells.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Maligna gliom är de vanligast förekommande hjärntumörerna hos vuxna. Trots behandling med kirurgi, strålbehandling och i vissa fall cytostatika (cellgift) är den förväntade överlevnaden kort. Meningeom är en form av hjärntumörer, som även om de anses vara godartade kan vara livshotande för patienten om den inte kan opereras bort.

En viktig förklaring till den dåliga prognosen hos hjärntumörer är att de är okänsliga för behandling med cytostatika pga multidrogresistens hos tumörcellerna. Viktiga faktorer som är inblandade i multidrogresistens är bl a P-glycoprotein (Pgp), multidrogresistens protein-1 (MRP1), lungresistens protein (LRP). Dessa proteiners normala funktion är bl a att transportera ut toxiska ämnen från cellerna. Reparation av DNA skador genom aktivering av enzymet O^6 metyl-guanin-DNA metyltransferas (MGMT) är också en viktig faktor till att tumörceller är okänsliga mot cytostatika. Patienter med elakartade gliom behandlas som regel med strålbehandling innan behandling med cytostatika ges. Det finns däremot få data på huruvida strålbehandling i sig påverkar uttryck av multidrogresistens.

EGFR -familjen (EGFR, ErbB2, ErbB3, ErbB4) är en samling proteiner som sitter på cellens yta och ger signaler in i cellen som bl a styr celltillväxt. Dessa proteiner är inblandade i utveckling av olika typer av cancer. Proteinuttryck av EGFR medför kortare överlevnad hos patienter med högmaligna gliom. Studier som rör de övriga receptorerna i EGFR-familjen (ErbB2, ErbB3, ErbB4) och deras kliniska betydelse i hjärntumörer är i stort sett okända. Proteinuttryck av EGFR ger signaler in till cellen som bl a leder till ökad celltillväxt och motverkar programmerad celldöd sk apoptos, vilket är en bidragande orsak till att tumörceller är okänsliga mot strålbehandling. Det kan därför vara kliniskt viktigt att motverka EGFR signalering för att få ökad effekt av strålbehandling.

Denna avhandling visar att pumpmekanismer som är involverade i multidrogresistens i kärlväggarnas celler i hjärnan aktiveras av strålbehandling. Dessa resultat kan därmed förklara den dåliga effekten av cytostatika när det ges efter strålbehandling. För att öka effekten av cytostatika i kombination med strålbehandling i maligna gliom, är det kanske nödvändigt att hämma aktiviteten hos drogresistenspumpar som sitter i kärlväggarnas celler, eller att ge cytostatika före strålbehandling.

En hittills okänd iakttagelse är en signifikant kortare överlevnad hos patienter med låggradiga gliom och högt uttryck av EGFR. Detta fynd, tillsammans med observationen att vissa låggradiga gliom har ett högt uttryck av Pgp, tyder på att det finns en mer malign undergrupp bland de tumörer som idag diagnostiseras som låggradiga gliom. Våra resultat visar därför på betydelsen av en mera individualiserad behandlingsstrategi, och att klassifikationssystem som idag används kan förbättras genom användandet av ytterligare biomarkörer.

Det höga proteinuttrycket av ErbB4 i låggradiga gliom jämfört med höggradiga gliom kan tyda på att ErbB4 fungerar som en reglerande gen i dessa tumörer.

Slutligen visar resultaten att det finns olika känslighet hos cellinjer vad det gäller effekt av protein signalhämmaren ZD1839 när denna drog ges tillsammans med strålbehandling. Resultaten visar också att tidpunkten för tillförsel av ZD1839 i relation till strålbehandling kan vara av stor betydelse för att optimera framtida kliniska studier där signalhämmare såsom ZD1839 utnyttjas för behandling av maligna gliom. Att drogen ges vid fel tidpunkt kan vara en förklaring till avsaknaden av positiva effekter när selektiva signalhämmare kombineras med konventionella behandlingstekniker som cytostatika och strålbehandling.

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