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In vivo expression profile of XIAP and Smac protein in gliomas and correlation with prognosis

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Abbreviations

aa	Amino acid
ACTH	Adrenocorticotropic hormone
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis inducing factor
Alk1, 4, 5	Activin receptor-like kinase 1, 4, 5
Apaf-1	Apoptotic protease activating factor 1
ARTS	Apoptosis-related protein in the TGF-beta signalling pathway
BAD	Bcl-2-antagonist of cell death
Bax	Bcl-2 associated x protein
Bcl-2	Protein family governing mitochondrial membrane permeabilisation, name derives from B-cell lymphoma 2
BID	BH3 interacting domain death agonist
BIR	Baculovirus inhibitory repeat
CD95	FAS ligand, member of the tumor necrosis factor family
c-FLIP	Cellular FADD-like IL-1β-converting enzyme-inhibitory protein
cIAP1	Cellular inhibitor of apoptosis protein 1
cIAP2	Cellular inhibitor of apoptosis protein 2
CNS	Central nervous system
DAB	3,3'-diaminobenzidine
DNA	Deoxyribonucleic acid
FADD	Fas-associated death domain
Fas	TNF receptor superfamily, member 6
GFAP	Glial fibrillary acidic protein
H&E	Haematoxylin and eosin stain
HtrA2/Omi	High temperature requirement protein A2
IAP	Inhibitor of apoptosis protein
IBM	IAP binding motif
IgG, IgM	Immunoglobulin G, M

ILPIP	hILP (XIAP)-Interacting Protein
IRES	Internal ribosome entry site
MIB-1	Made in Borstel-1 (town in Schleswig-Holstein) monoclonal antibody for Ki-67 (Kiel-67), a proliferation marker
ML-IAP	Melanoma inhibitor of apoptosis
mRNA	Messenger ribonucleic acid
Myc	Myelocytomatosis oncogene, family of transcription factors
NAIP	Neuronal apoptosis inhibitory protein
PBS	Phosphate buffered saline
PTEN	Phosphatase and tensin homolog
RING	Really interesting new gene
TAK1	TGF- activated kinase 1
TMA	Tissue microarray
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
VEGF	Vascular endothelial growth factor
WHO	World health organisation
XAF1	XIAP associated factor-1
XIAP	X-linked inhibitor of apoptosis protein

1 INTRODUCTION

1.1 Apoptosis

1.1.1 Overview

Apoptosis, also referred to as programmed cell death, is an evolutionary conserved, intrinsic process by which cells systematically disassemble and degrade their own cellular components. It plays an important role in the embryological development of multi-cellular organisms, the regulation of hormone dependent and cyclically stimulated tissues, the function of the immune system, the removal of cells with irreparable genotoxic damage and the general steady state turnover. Several ways of apoptosis induction have been described. It can be activated by genetically defined developmental programs, by endogenous signal transducing proteins, cytokines and hormones but also by extrinsic means, such as genotoxic therapy, oxidative stress and hypoxia. The capability of a cell to undergo apoptosis in response to a death signal is dependent on its proliferative status, cell cycle position and the controlled expression of genes that promote and inhibit programmed cell death. Stringent regulation of these death modulating factors must be maintained to ensure that apoptosis occurs in the proper physiological context. Defects at any point within the death pathway may result in inappropriate apoptosis, potentially leading to pathophysiological conditions *(Webb et al., 1997)*.

1.1.2 Historic background

The term apoptosis was originally coined by Kerr, Wyllie and Currie in 1972 following their observations of cellular events in the adrenal cortex after ACTH deprivation *(Kerr et al., 1972)*. The expression is Greek in origin (*apo* = from and *ptosis* = falling) and translates roughly as ‘falling leaves’. Among other features they tried to express the detachment of the apoptotic cells akin to the falling of leaves in autumn (for the morphology of apoptosis, see 1.1.3). The term programmed cell death on the other hand is a few years older and was probably first used in a doctoral thesis by Richard Lockshin in 1963 referring to observations in the muscles of silk moths *(Lockshin, 1963)*.

Long before apoptosis was reincarnated in our times, naturally occurring cell death was a prospering scientific subject at the end of the 19th century. Carl Vogt was the first to report that cells die naturally in the development of the midwife toad in 1842 very shortly after the establishment of the cell theory by Schwann in 1839 *(Vogt, 1842; Schwann, 1839)*. Beard's discovery of neuronal cell death in fish embryos in 1889 was another landmark of apoptosis research *(Beard, 1889)*. Collin, who described motoneuron and spinal ganglion cell death in development, was the first who understood the numerical importance of this phenomenon and argued that an overproduction of cells followed by degradation might be a concept in all tissues *(Collin, 1906)*. The first clear morphological description of the apoptotic phenotype was by Flemming in 1885 describing the natural regression of ovarian follicles in mammals (*Flemming, 1885)*. He called his observation 'Chromatolysis' and the term was widely used for the next 30 years. The ebbing of interest in cell death after the year 1914 seems to be at least partly due to the fact that most reports up to that time were written in German, a language an increasing proportion of scientists were unable to read *(Clarke and Clarke, 1995).*

1.1.3 Sequence and morphology of apoptosis

A cell undergoing apoptosis runs through a sequence of three distinct stages. The first stage begins with the commitment of the cell to undergo apoptosis after the cell has received a lethal apoptotic stimulus. The time frame of this phase is variable and dependent on cell type, apoptotic stimulus, position in the cell cycle and expression of death modulating factors *(Webb et al., 1997)*. The second stage is the execution stage, the phase of apoptosis in which the major morphological changes occur. The entire process of the second phase is completed within a few hours and this time is relatively constant in relation to cell type and apoptotic stimulus *(Bursch et al., 1990)*. Kerr et al. were the first to systematically describe the structural changes of the execution stage *(Kerr et al, 1972)*, while many others have since then helped to extend the understanding of the processes underlying this phase. One of the hallmark changes of cell morphology is the condensation of chromatin and its aggregation along the nuclear periphery, the cause of this being an ordered degradation of the DNA

by endogenous nucleases into nucleosomal fragments of 180-200 base pairs *(Wyllie et al., 1980)*. At the same time the ultrastructure of the nucleus is altered by the proteolytic cleavage of the intermediate filament network of the nuclear lamina leading to fragmentation of the nucleus into chromatin containing fragments *(Kaufmann, 1989; Lazebnik et al., 1993; Ucker et al., 1992)*. While other cytoplasmatic organelles remain structurally intact, a loss of mitochondrial transmembrane potential associated with a dysfunction of mitochondria has been observed *(Kroemer et al., 1995)*. Cytoplasmic proteins are inactivated by cross-linking while cytoskeletal filaments aggregate in parallel structures *(Fesus, 1993)*. The loss of structural proteins in the cytoplasm is followed by a rounding and shrinking of the cell. Changes of the structural integrity of the plasma membrane cause the formation of protuberances that ultimately separate from the main body thereby forming membrane encased apoptotic bodies.

The changes of the plasma membrane are particularly important for the third stage of apoptosis, the phase of phagocytotic clearance. This stage is of especial significance to the central strategy of apoptosis, as it allows the removal of cells without the disruption of the plasma membrane and leakage of intracellular proteins, thereby preventing an inflammatory response. Apoptotic cells and apoptotic bodies are rapidly recognized and phagocytosed by nearby cells or macrophages in the surrounding tissue. The recognition is dependent on several surface structures on both macrophages and apoptotic cells and involves the interaction of macrophage lectins with specific N-acetyl sugar molecules on the surface of apoptotic cells, receptor mediated interactions with phosphatidylserine in the outer layer of the plasma membrane and thrombospondin/cell surface receptor interactions *(Savill and Fadok, 2000)*.

1.2.1 Apoptosis in cancer

Deregulation of apoptosis has been observed in a large array of pathological settings. Generally speaking, this deregulation can take two forms, either an increase of cellular sensitivity towards apoptotic stimuli, resulting in excessive cell death as observed in neurodegenerative diseases, vascular diseases and AIDS or a resistance to apoptosis as expected in benign and malignant

neoplasia, autoimmune disorders and chronic/latent viral infections *(Thompson, 1995)*

Many forms of neoplasia or cancer are characterized by an activation of proliferative pathways. Experiments measuring the doubling time and length of the cell cycle in malignant tumors have yielded some interesting results, as in some tumors the replication rate was even lower than in their tissue of origin and the duration of the cell cycle is usually longer *(Nowell, 1990)*. This clearly indicates that increased proliferation is not the only explanation for tumor cell gain and that a reduction of physiological cell loss is another major factor in this process *(Benitez-Bribiesca, 1998)*. The first concrete example of an oncogene that acts by inhibiting apoptosis rather than by enhancing proliferation was the forced overexpression of Bcl-2 protein in various haematologic cells *(Vaux et al., 1988)*. Further studies could show that proliferation-inducing oncogenes such as Myc cooperate with other oncogenes like Bcl-2 and that this interaction is necessary for malignant cell transformation *(Cory et al., 1999; Fanidi et al., 1992)*. Ex vivo experiments on the adenoma to carcinoma axis in colon tissue demonstrated a reduction of apoptosis with an increase of malignancy and subsequent studies in other neoplasms have yielded comparable data *(Bedi et al., 1995)*. In recent years several other regulatory proteins of apoptosis have been shown to be deregulated in neoplasia, predominantly members of the IAP family which are highly expressed in several tumors *(Nachmias et al., 2004)*.

1.2.2 Apoptosis in astrocytoma

For the purpose of this study a group of tumors deriving from cells of the astrocytic lineage was investigated. Astrocytes are glial cells and have traditionally been viewed as passive elements in the CNS, providing structural and metabolic support to neurons but playing little role beyond this. New results show, that the function of astrocytes has been grossly underestimated. The micro-regulation of cerebral blood flow, communication of astrocytes with other glial cells through intracellular waves of calcium or via intercellular diffusion of chemical messengers and even the induction of excitatory potentials in nearby neurons have recently been attributed to these cells *(reviewed in Fields and*

Stevens-Graham, 2002; Harder et al., 2002, Schipke and Kettenmann; 2004). Even the traditional morphology of the astrocyte, as traditionally determined by its expression of GFAP, has recently been revised, indicating how little is known of this cell lineage (Figure 1) *(Bushong et al., 2002)*.

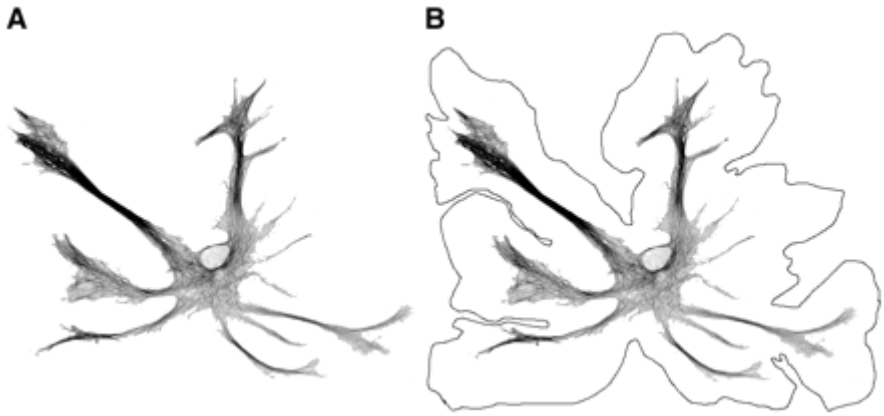

Figure 1. Illustration of a single astrocyte. (A) Staining of glial fibrillary acidic protein (GFAP) while (B) shows actual cell outlines (image from *Bushong et al., 2002*).

Astrocytomas are the most frequent neoplasms of the brain and account for more than 60% of all primary brain tumours. Several attempts at classification have been performed during the years of neuropathological research, currently the WHO-classification is the one mostly used. In this classification the astrocytic tumors are subdivided into four grades (WHO I-IV) primarily by histological criteria. These grades are of high clinical importance, as they have proven to be strongly correlated to patient prognosis *(Kleihues and Cavenee, 2000)*.

The following paragraph gives an overview of the biology and histology of the tumors investigated in this study with a highlight on what is known of the prevalence of apoptosis in these entities.

The pilocytic astrocytoma WHO grade I

The pilocytic astrocytoma is a tumor of the young with most cases occurring in the first two decades of life. It predominantly manifests in mid-line structures of the CNS, often in the cerebellum, the brain stem or the optic nerve *(Kleihues and Cavenee, 2000)*. It has the most favorable prognosis of all astrocytomas with a 10 year survival of 96% *(Ohgaki and Kleihues, 2005)*. Several prognostic factors have been identified, including the localization of the tumor, patient age at diagnosis, the radicalism of the primary operation and histological subgroups (classic vs. mixed pilocytic astrocytoma) *(Haapasalo et al., 1999)*. Pilocytic astrocytomas generally show a circumscribed growth pattern clearly distinguishing them from higher grade gliomas with a diffuse infiltration. In histopathology, these tumors classically exhibit a biphasic pattern in which compact regions of bipolar and highly fibrillated astrocytes accompanied by Rosenthal fibers alternate with microcystic regions with a loose cell compound. An interesting histological aspect of these tumors is the possible presence of endothelial proliferation, defined as a definite multilayering of the endothelium. The absence of atypia in the spindle shaped cells, the lack of mitoses and necroses and the molecular genetic characteristics reflect the favorable nature of these tumors. Importantly, pilocytic astrocytomas usually do not contain genetic changes known to be associated with the progression of astrocytomas of the diffuse infiltrating type *(von Deimling et al., 1994)*.

Several studies have so far elucidated the prevalence of apoptosis in pilocytic astrocytoma. Kordek et al. reported very low apoptotic ratios of 0.006% (+/- 0.008% standard deviation; range, 0-0.02) but only investigated a small array of pilocytic astrocytomas (n=9) using in situ nonradioactive tailing of DNA breaks (TUNEL) *(Kordek et al., 1996)*. These results were confirmed by Haapasalo et al. in 1999 in a larger array of cerebellar pilocytic astrocytoma (n=46), although here the apoptotic ratios were rather higher (mean 0.03%, +/-0.10% standard deviation), probably due to the selection of tumor areas with the highest expression for analysis *(Haapasalo et al., 1999)*. In agreement, flow cytometrical analysis also showed that both spontaneous and radiation-induced apoptosis levels are very low in pilocytic astrocytoma *(Hassounah et al., 2005)*.

Nakamizo et al. have yielded considerably higher results in a small array of tumors (n=6) with apoptotic ratios of 17.9% (+/- 5.16% standard deviation) also using the TUNEL assay *(Nakamizo et al., 2002)*. Another early descriptive study of apoptosis in 2 pilocytic tumors also exhibited high apoptotic ratios with a mean of 8.8% TUNEL positive cells *(Patsouris et al., 1996)*. Especially the results of the latter two publications emphasize the importance of morphological confirmation in apoptosis detection, as an inadequate correlation with the apoptotic phenotype might lead to such high results. Darzynkiewicz states that regardless of the assay used, the mode of cell death should generally be positively identified by morphological microscopic examination and this should be the deciding factor in situations of ambiguity *(Darzynkiewicz et al., 1992)*. In personal communication with Darzynkiewicz via e-mail on this specific discrepancy in pilocytic astrocytoma he commented that low levels in apoptotic ratios are often due to the failure of optimization of the staining protocol, >> *in other words "the lack of evidence is not the evidence of the lack". On the other hand such high estimate as 17% seems unlikely – apoptosis is a kinetic event of rather short duration and apoptotic cells "disappear" rather rapidly, through shedding apoptotic bodies and being ingested by neighboring cells. Thus, even if incidence of apoptosis is high, AI* (Apoptotic Index; as defined by *Staunton and Gaffney, 1995*) *remains rather low << (Darzynkiewicz, 2006).*

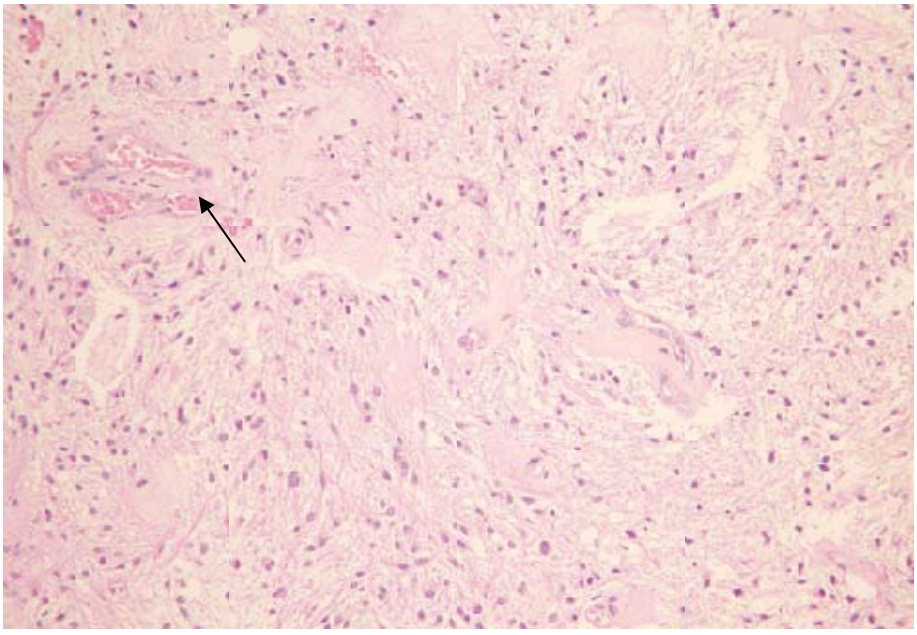

Figure 2. Pilocytic astrocytoma WHO I of an 14 year old girl with endothelial proliferation (arrow, H&E stain, original magnification 200x).

WHO grade II diffuse astrocytoma

Diffuse astrocytoma WHO II are tumors of the middle age with a peak incidence of 30 to 40 years of age. The entities' most evident difference to the pilocytic astrocytoma is its diffuse growth into normal brain tissue, often with a broad infiltration zone. Thus, a complete resection of these tumors is generally not possible, resulting in a very high degree of tumor relapses and a worse prognosis. Median survival after the first operation is 5.6 years. Factors influencing this time are patient age, tumor histology and the presence of a neurological deficit *(Ohgaki and Kleihues, 2005; Wessels et al., 2003)*. Three histological subtypes of astrocytoma WHO II exist, the most frequent fibrillary astrocytoma, the gemistocytic astrocytoma and the very rare protoplasmatic form. Because of the drastically reduced survival of the gemistocytic astrocytoma compared to other low grade tumors future editions of the WHO classification are likely to assign the grade III to these tumors *(Ohgaki and Kleihues, 2005)*. Hybrid forms also exist. All of these forms share a low to moderate increase of cellular density, a low grade of nuclear atypia, and the absence of mitoses and necrosis *(Kleihues and Cavenee, 2000).* A hallmark in genetic alteration in low grade diffuse astrocytoma is the mutation of the TP53 gene, a gene encoding a transcription factor that is activated in response to a variety of DNA-damaging agents. Activation of TP53 results either in a G_1 cell cycle arrest or in apoptosis and thereby contributes to the suppression of malignant transformation and the maintenance of genomic integrity *(Kastan et al., 1995)*. Especially the gemistocytic astrocytoma exhibits a high percentage of mutation of around 88% compared to 53% in fibrillary astrocytoma *(Ohgaki and Kleihues, 2005)*.

Apoptotic ratios in these tumors are generally low. Kordek et al. and Ralte et al. both observed comparable results (0.1% SD 0.1 [n=18] and 0.14% SD 0.18 [n=30]), while Nakamizo again yielded considerably higher ratios (3.96% SD 3.57 [n=14]) *(Kordek et al., 1996; Nakamizo et al., 2002; Ralte et al., 2001)*.

Heesters et al. investigated 28 astrocytomas and found apoptotic ratios akin to first two groups (0.3% and 0.5% for astrocytomas with a gemistocytic component; *Heesters et al., 1999*).

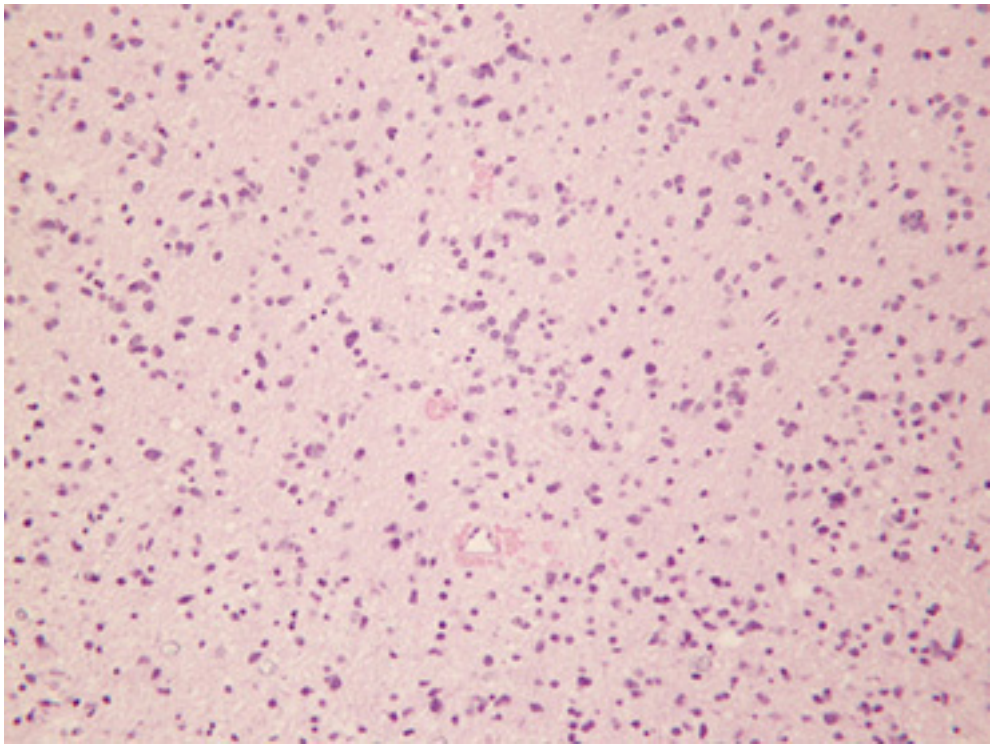

Figure 3. Fibrillary astrocytoma of a 33 year old male (H&E stain, original magnification 400x). The tumor demonstrates a moderate increase of cellular density, a low grade of nuclear atypia, and no mitoses or necrosis.

Anaplastic astrocytoma WHO III

Anaplastic astrocytomas have their peak of incidence at 45 years of age with an broad distribution mostly ranging from 30 to 60 years. Median survival of these aggressive tumors is as low as 1.6 years *(Ohgaki and Kleihues, 2005).* Consistently reported influence factors on survival are age at operation, Karnofsky performance status and evidence of an oligodendroglial component of the tumor *(Chang et al., 2001; Kallio et al., 1991; Kleihues and Cavenee, 2000)*. In several respects anaplastic astrocytomas take an interposing position between diffuse low grade tumors and the glioblastoma. Tumor progression can advance a low grade astrocytoma to the anaplastic form and anaplastic tumors can also progress to the glioblastoma, though low grade tumors can also omit the anaplastic form and progress directly to grade IV. Mean cellular density is higher than in low grade tumors but is still below the density in glioblastomas. Grade III tumors show a high degree of nuclear pleomorphism. The WHO classification permits sporadic mitoses as well as beginning vascular endothelial

proliferation. Necrosis is generally not a common feature in anaplastic astrocytoma and it is never seen in the typical pseudopalisading form of necrosis as in glioblastoma *(Kleihues and Cavenee, 2000)*.

The levels of apoptosis in anaplastic astrocytoma previously reported were mostly under 1%, though most of these studies suffer from small patient samples (0.3% +/- 0.3% SD [n=10]; 0.07% +/- 0.05% SD [n=11], 0.7% [n=7]) *(Kordek et al., 1996; Patsouris et al., 1996; Ralte et al., 2001)*. The largest study sample was comprised of 29 tumors and found similarly low levels of 0.8% TUNEL positive cells *(Heesters et al., 1999)*. In another small sample (n=9) Nakamizo demonstrated apoptotic ratios just above 1% *(Nakamizo et al., 2002)*.

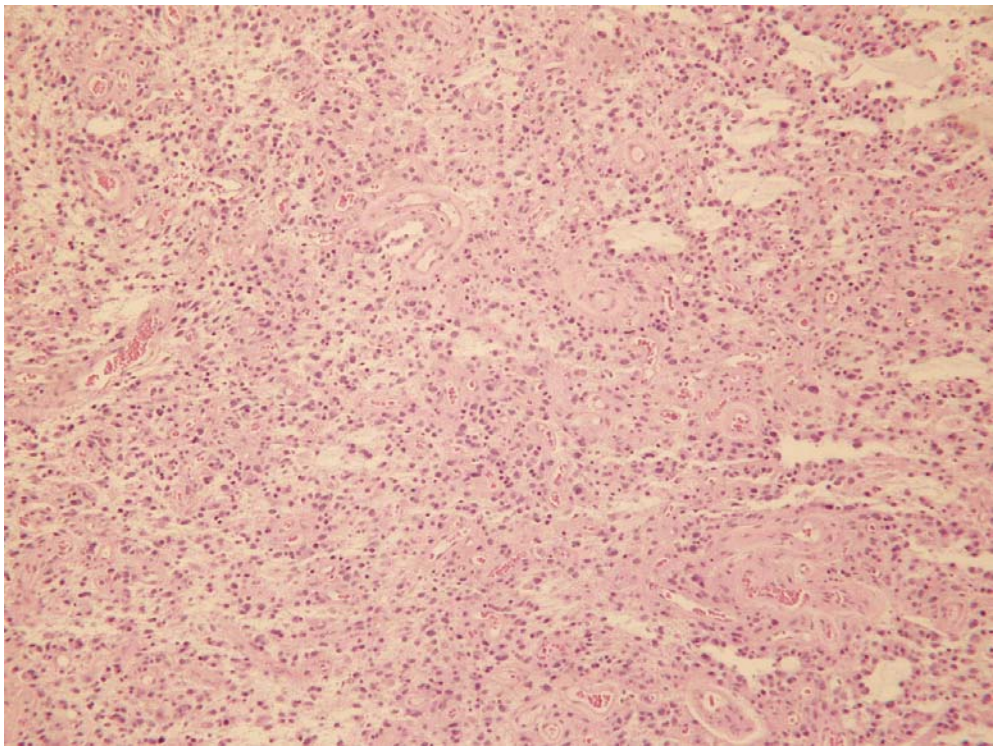

Figure 4. Anaplastic astrocytoma of a 36 year old male (H&E stain, original magnification 200x). Mean cellular density and degree of nuclear pleomorphism are higher than in low grade tumors. As in this illustration, mitoses are often scarce, complicating the differentiation of grade II and III tumors. Necrosis is generally not a common feature of grade III.

Glioblastoma WHO grade IV

The glioblastoma is the most aggressive and at the same time most frequent primary tumor of the CNS. A recent epidemiological study revealed an incidence rate of 3.55 new cases per 100 000 persons per year (adjusted to the European Standard Population). In the same study a median survival time of 4.9 months was demonstrated and 3 year survival rates of patients at the population level were as low as 1.2%. This is far below the usual median survival time of around one year. This was explained by the fact that in the study group over 30% of the patients were over 70 years of age and only 54% of the glioblastoma patients underwent surgical intervention. The higher survival times of clinical studies is probably due to an unnatural selection of younger and fitter patients *(Ohgaki and Kleihues, 2005)*. The highest incidence of glioblastomas is between 49 and 75 years, though rare cases of glioblastoma in

childhood also exist. The preferential localization of the glioblastoma is the white matter of the frontal and parietal lobe. Glioblastomas exhibit a highly infiltrative growth and infiltrating cells found several centimeters from the brain/tumor interface complicate the tumor demarcation, thereby limiting the means of surgical eradication *(Giese et al., 2003).* Tumors of this type may also infiltrate across the corpus callosum, producing a so called butterfly glioma. Multifocal lesions have been reported to represent 1% to 10% of the cases at the initial presentation *(Barnard and Geddes, 1987)*. In about 95% of cases glioblastomas present with no precursor lesion, only 5% are secondary glioblastoma progressing from known lower grade tumors *(Ohgaki and Kleihues, 2005)*. Histological hallmarks of the glioblastoma are a high cellular density, a high degree of nuclear pleomorphism, the frequent mitoses, the pseudopalisading form of necrosis and multilayered endothelial proliferation (also called microvascular hyperplasia or glomeruloid vascular proliferation). Genetic alterations of an individually varying degree have been detected in glioblastoma proposing the existence of several genetic subgroups.

The levels of apoptosis in glioblastomas are often slightly higher than in other diffuse astrocytoma and range from 0.17% (+/- 0.19 SD, n=15) to 2.1% (+/- 1.6 SD, n=10) *(Nakamizo et al., 2002; Ralte et al., 2001)*. In the study by Kordek the increase from low grade to high grade astrocytoma (glioblastoma and anaplastic astrocytoma) reached significance *(Kordek et al., 1995)* and similar results were observed by Caroll, and here even the increase from grade III to grade IV was significant *(Carroll et al., 1997)*. The largest available study was on 136 glioblastomas and showed a mean TUNEL staining of 1.7%. Here, glioblastoma also had significantly more apoptosis than other diffuse astrocytic tumors *(Heesters et al., 1999)*. In the work by Nakamizo the highest levels of apoptosis in all WHO grades were observed in grade I tumors with a significant difference to all higher grade tumors *(Nakamizo et al., 2002)*.

The debate whether a higher level of apoptosis predicts a favorable outcome in astrocytic tumors has still not come to an end, though the scales are tipping towards the side that spontaneous apoptosis has no direct association with survival. The level of apoptosis also does not correlate with several factors

linked to apoptosis regulation like p53, PTEN, or Bcl-2 *(Carroll et al., 1997; Kordek et al., 1996; Nakamizo et al., 2002)*. As the levels of apoptosis and mitosis correlate at least in the whole group of astrocytomas, the theory has been stated that the high levels of apoptosis in glioblastoma might be due to a more prominent tumor hypoxia and may therefore be a marker for a high selective pressure on the tumor cells *(Steinbach and Weller, 2004)*.

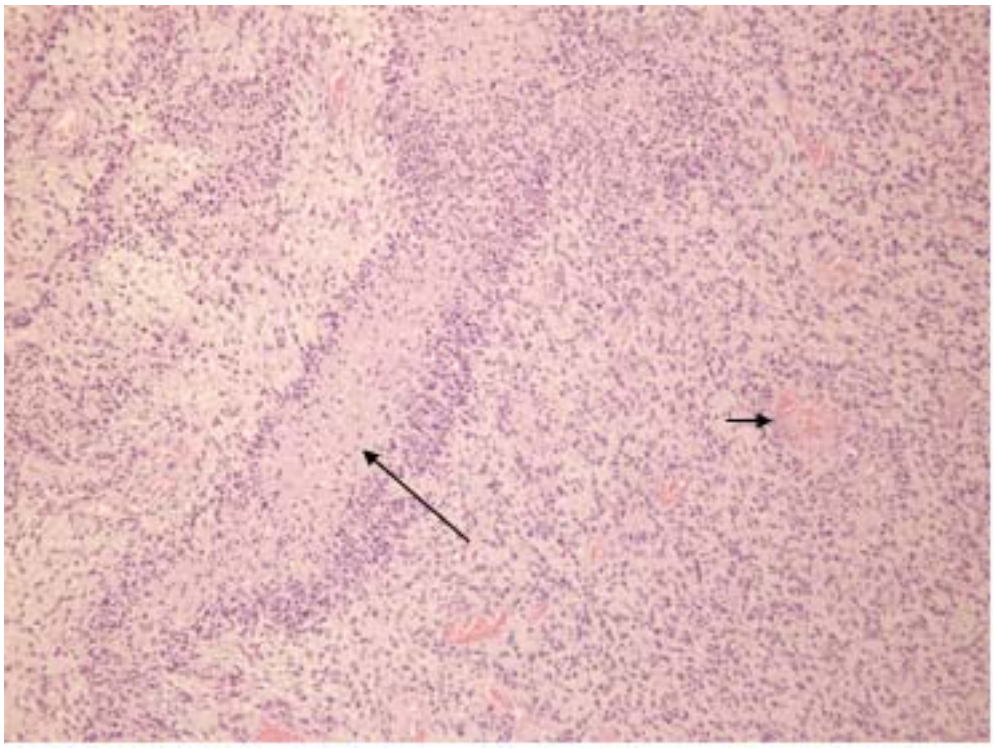

Figure 5. Glioblastoma WHO grade IV of a 51 year old male patient displaying high cellular density, typical pseudopalisading necrosis (arrow long) and microvascular hyperplasia (arrow short, H&E stain, original magnification 100x)

1.3.1 Molecular effectors of apoptosis

In recent years a family of cystein proteases termed the caspases has emerged as the central executioners of apoptosis. The first of these proteins was found as a major regulator of apoptosis in the nematode Caenorhabditis elegans *(Ellis and Horvitz, 1986)* and since then over a dozen caspases have been identified in humans *(Earnshaw et al., 1999; Thornberry and Lazebnik, 1998)*. Because of confusion concerning the various names of the caspases in the early years of apoptosis research a proposal to name the caspases in order of their first

publication has generally achieved acceptance *(Alnemri et al., 1996)*. The human caspases share the following structural and functional characteristics: (i) they all induce apoptosis when overexpressed, (ii) they are all expressed as enzymatic inert zymogens and have to be processed proteolytically to function, (iii) all but three (caspase-8, -9 and -10) share a conserved pentapeptide sequence QACRG as the active site (iv) and they all cleave their substrate after an aspartic acid residue *(Philchenkov, 2003).*

Functional differences between the various caspases exist, most importantly the activation of the various caspases and their preferred substrates. Three general ways of caspase activation have been described so far. (i) Caspase-8 is the key initiator caspase in the death-receptor pathway and is activated by a proximity induced mechanism. The binding of death receptors such as CD95 (Apo-1/Fas) leads to the formation of intracellular signaling complexes, which then recruit several molecules of procaspase-8. As these proenzymes already possess a weak intrinsic protease activity, their high local concentration is sufficient to cleave and activate one another. (ii) The procession by upstream caspases is used extensively for the activation of the three short prodomain caspases-3, -6 and 7. These have also been termed effector caspases as they are responsible for the degradation of vital cellular proteins, important in processes like DNA repair, macromolecular processing, cytoskeletal integrity and cell signaling *(Brancolini et al.,1995; Casciola-Rosen et al., 1994; Casciola-Rosen et al., 1995; Emoto et al., 1995; Kayalar et al., 1996; Lazebnik et al., 1994; Lazebnik et al., 1995; Wang et al., 1996b*). This cascade of caspase activation can be seen as a useful method to amplify and integrate pro-apoptotic signals. (iii) The third method of caspase activation has so far only been described for caspase-9. This caspase associates in a complex with the two cofactors Apaf-1 and mitochondrial cytochrome c, thereby forming the apoptosome that cleaves and activates downstream effector caspases such as caspase-3 *(Cain et al., 1999; Li et al., 1997; Zou et al., 1999)*.

The above observations lead to the proposal of two distinct pathways of apoptosis induction, the extrinsic pathway mediated by death receptors *(Ashkenazi and Dixit, 1998)* and the intrinsic pathway via mitochondrial factors

(Green and Reed, 1998). Further studies could show that several points of crosstalk between the two pathways exist, as activators of the death receptor pathway like TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand) initiate both, the extrinsic and the intrinsic pathway of apoptosis induction *(Fulda et al., 2001; Song et al., 2003)*.

Another family of proteins demonstrated importance in the regulation of the intrinsic activation of apoptosis. The Bcl-2 family is comprised of members with anti-apoptotic activity and of members that promote programmed cell death. Several forms of cellular stress shift the ratio of pro-apoptotic and anti-apoptotic members of the Bcl-2 family in favor of the pro-apoptotic factors like Bax, BID and BAD *(Cory et al., 2003; Oltvai et al., 1993; Wang et al., 1996a)*, thereby leading to the perturbation of the mitochondrial membrane and the release of cytochrome c, Smac/DIABLO, HtrA2/Omi, Endonuclease-G, AIF, ARTS and possibly others which form the arsenal of the mitochondria to promote apoptosis *(Du et al., 2000; Kluck et al., 1997; Larisch et al., 2000; Li et al., 2001; Susin et al., 1999; Suzuki et al., 2001a; Verhagen et al., 2000)*. While Endonuclease-G and AIF translocate to the nucleus and induce DNA fragmentation *(Cande et al., 2004; Li et al., 2001)*, cytochrome c forms a pro-apoptotic complex with Apaf-1 and caspase-9 (see above). Smac/DIABLO, HtrA2/Omi and ARTS bind and thereby inhibit members of the Inhibitor of Apoptosis Protein (IAP) family.

Expediently termed Inhibitor of Apoptosis Proteins, this family is conserved from baculovirus to man and performs at a crucial point of apoptosis control. By inhibiting the terminal effector caspases, the IAP members exhibit an intrinsic decisive role in the regulation of apoptosis *(Deveraux and Reed, 1999; Philchenkov, 2003)*. The family shares a conserved baculoviral inhibitor repeat (BIR) domain capable of binding and inhibiting active caspases. For c-IAP1, c-IAP2, XIAP and survivin an inhibition of active caspase-3 and -7 has been demonstrated. As these caspases function as the executioners of the intrinsic and the extrinsic pathway of apoptosis their inhibition permits control over a wide range of apoptotic stimuli.

1.3.2 XIAP

XIAP (X-linked Inhibitor of Apoptosis Protein) is a highly conserved protein of 497 amino acids assigned to Xq25 and is translated by a cap-independent mechanism of translation initiation which is mediated by a unique internal ribosome entry site (IRES). This allows XIAP mRNA to be actively translated during conditions of cellular stress when the majority of cellular protein synthesis is inhibited *(Holcik et al., 1999; Rajcan-Separovic et al., 1996)*.

Functions of XIAP

The protein was first described as a potent and direct inhibitor of caspase-3, -7 and -9 *(Deveraux et al., 1997; 1998; 1999)* and also blocks Fas-mediated apoptosis in glioma cells *(Wagenknecht et al., 1999)*. In some experimental settings XIAP was shown to be the most potent inhibitor of caspases *(Deveraux and Reed, 1999; Roy et al., 1997)*. Like other IAPs capable of binding caspases, XIAP contains three conserved approximately 80 amino acid Baculoviral Inhibitor of apoptosis Repeat (BIR) motifs, followed by a RING zinc finger domain *(Deveraux and Reed, 1999)*. Surprisingly, it has been shown that the BIR regions are dispensable for the inhibition of caspase-3 and caspase-7 *(Chai et al., 2001)*. Further evaluation of the crystal structure identified the conserved linker region preceding BIR2 to be the binding site of caspase-3 and -7, while the BIR2 domain itself serves as a regulatory element for caspase binding *(Huang et al., 2001; Riedl et al., 2001; Sun et al., 1999).* A specific binding site for caspase-9 was found within BIR3 *(Deveraux et al., 1999; Sun et al., 2000)*. The BIR3 domain forms a heterodimer with a caspase-9 monomer, thus preventing the formation of the catalytic active dimer form of caspase-9 *(Shiozaki et al., 2003)*.

Further roles in pathways directly or indirectly connected to apoptosis have also been described, including a modulation of the transforming growth factor-ß signaling through Alk1, Alk4 and Alk5 *(Birkey Reffey et al., 2001)*, interference with c-jun-NH_2-kinase signaling by TAK1 and ILPIP *(Sanna et al, 1998; 2002a; 2002b; Yamaguchi et al., 1999)* and TAK1-dependent activation of nuclear

factor-κB *(Hofer-Warbinek et al., 2000)*. The direct interaction of XIAP with these central pathways of apoptosis moves XIAP into an orchestrating position of apoptosis regulation far beyond the sole inhibition of terminal caspases.
Other functions of XIAP include ubiquitin ligase activity *(Morizane et al., 2005)* and a newly described transient interaction of the BIR3 domain with checkpoint kinase (Chk) 1 during mitosis. The latter may imply a mechanism of coupling between the regulatory networks that control cell cycle progression and apoptosis *(Galvan et al., 2004)*.
Despite its numerous interactions, XIAP-deficient mice showed no abnormalities; the only difference detectable was an increased level of other IAP proteins (c-IAP1 and c-IAP2). This suggests that a compensatory mechanism exists leading to the upregulation of other IAP family members when XIAP expression is lost *(Harlin et al., 2001)*.

Regulators of XIAP
Several endogenous negative regulators for XIAP exist, further indicating its important position in apoptosis *(Holcik et al., 2001)*. The nuclear factor XAF1 (XIAP Associated Factor 1) interacts with XIAP and thereby possibly regulates the endogenous balance of pro- and anti-apoptotic drive during normal cell life. In vitro overexpression triggered a redistribution of XIAP from the cytoplasm to the nucleus and neutralized its ability to inhibit cell death *(Fong et al., 2000; Liston et al., 2001)*. Smac/DIABLO, HtrA2/Omi and ARTS are released from the mitochondria after the initiation of apoptosis and then bind and thereby block the function of XIAP to inhibit the terminal caspases *(Du et al., 2000; Larisch et al., 2000; Suzuki et al., 2001a; Verhagen et al., 2000).*
During Fas and TRAIL induced apoptosis XIAP is cleaved at amino acid 242 into two fragments, separating the BIR1 and 2 domains from the BIR3 and RING domains. This considerably reduces XIAPs potency to inhibit apoptosis and may be another mechanism by which cell death programs circumvent the anti-apoptotic barrier posed by XIAP *(Deveraux et al., 1999; Song et al., 2003)*.

XIAP in glioma

In many glioma cell lines a high expression of XIAP mRNA and protein has been demonstrated *(Wagenknecht et al., 1999)* and has later been found to be at least partly responsible for the apparent resistance of gliomas to available chemotherapy *(Grossman and Batara, 2004)*, but also to recent approaches focusing on the death ligand TRAIL *(Roa et al., 2003)*. A multitude of experiments has been performed on glioma cells displaying the effectiveness of lowering XIAP levels and thereby enhancing the cells susceptibility towards apoptotic stimuli *(Fulda et al., 2002a; Hatano et al., 2004; Kim et al., 2004; Li et al, 2004; Schimmer et al., 2004)*.

1.3.3 Smac

Smac is synthesized as a 239 amino acid precursor protein, with the NH_2-terminal 55 amino acids serving as a mitochondrial-targeting signal. Its encoding region was located to chromosome 12q24 *(Du et al., 2000; Verhagen et al., 2000)*. The mechanism of Smac release from the mitochondria is still controversially discussed, but it is described to be caspase dependent and Smac is translocated within a time frame of 19 +/-3 minutes after apoptosis initiation *(Springs et al., 2002; Adrain et al., 2001).*

Functions of Smac

Once released into the cytosol Smac binds to XIAP and thereby disrupts its function to block caspase-3, caspase-7 and caspase-9 *(Chai et al., 2000; Huang et al., 2003; Srinivasula et al., 2000; Srinivasula et al., 2001; Wu et al., 2000)*. Smac has also been described to inhibit several other members of the IAP family including c-IAP1, c-IAP2, ML-IAP and survivin but not NAIP, the neuronal apoptosis inhibitory protein *(Davoodi et al., 2004; Sun et al., 2005; Vucic et al., 2002)*. For an overview of the following modes of interaction of Smac and its iso-types with XIAP see Figure 6 below.

Smac directly interferes with the interaction of the BIR3 domain of XIAP and caspase-9 utilizing its 4 N-terminal amino acid residues (Ala-Val-Pro-Ile). This region forms a highly conserved IAP binding motif (IBM) which masks the

caspase-9 binding site on a hydrophobic pocket in the BIR3 domain *(Srinivasula et al., 2001; Wu et al., 2000)*. The proximate amino acids (aa 5~30) sterically inhibit the binding of caspase-3 and caspase-7 to the linker region near BIR2 of XIAP while the subsequent amino acids through to the C-terminus are described as an extensive hydrophobic interface, triggering Smac homodimerization and thereby further enhancing its anti-apoptotic function *(Chai et al., 2000; Huang et al., 2003; Srinivasula et al., 2000)*.

A further mode of interaction of Smac and XIAP was found to function via the RING domain located near the C-terminal of XIAP. This region confers ubiquitin protease ligase (E3) activity to the protein and was shown to be important in XIAP auto-ubiquitination and degradation *(Yang and Li, 2000)*. It has also been verified that the isoform Smac3/Smac δ, but not Smac/DIABLO, accelerates XIAP auto-ubiquitination and destruction and an intact RING domain of XIAP is necessary for this *(Fu et al., 2003; Roberts et al., 2001)*. Experiments on thymocytes revealed that RING auto-ubiquitination and degradation of IAPs may be a key event in the apoptotic program *(Yang et al., 2000).* Interestingly, the XIAP RING domain is also active in the ubiquitination of Smac and other key proteins of apoptosis like caspase-3 *(MacFarlane et al., 2002; Suzuki et al., 2001b).*

The interaction of XIAP with Smac beta/Smac-s, the recently discovered third isoform of Smac which lacks the mitochondrial targeting sequence and the IBM raises several conceptual questions. The isoform was found to be expressed in the cytosol of several cancer cell lines and is capable of releasing caspase-3 and caspase-7 inhibition, corresponding to the homologous aa 5-30 of Smac/DIABLO *(Srinivasula et al., 2000).* Later studies indicate that Smac beta/Smac-s is physiologically processed in the cytosol, thereby disrupting its ability to bind XIAP and to release caspase inhibition. Interestingly, the function to induce apoptosis was unchanged, suggesting that the release of caspase inhibition might not be the main pro-apoptotic aspect of Smac and its iso-types *(Roberts et al., 2001)*. This point is further supported by the fact that XIAP's anti-apoptotic function is dependent on its binding of Smac while its function to bind caspases is dispensable. Thus it is possible that Smac does not bind to

XIAP to enhance caspase activity, rather XIAP binds to Smac to inhibit its further, as yet unknown, apoptosis-inducing functions *(Silke et al., 2002)*. The issue remains controversial, as other results exist clearly showing that the deletion of the IAP recognition motif of Smac completely suppressed sensitization to etoposide-mediated killing *(Hunter et al., 2003)*.

To date, there is little proof of the relevance of Smac under physiological conditions in vivo, as Smac-deficient mice (Smac[-/-]) were able to compensate the absence of Smac by an unknown mechanism and showed no abnormalities *(Okada et al., 2002)*.

Smac in Glioma

While no data on the expression of Smac protein in gliomas is available, several studies demonstrated that Smac or mimics of its function potentiate the anti-tumor effects of various genotoxic therapies in glioma cell lines *(Giagkousiklidis et al., 2005; Li et al., 2004; Mizukawa et al., 2006)*. Additionally, a human glioma xenograft model in nude mice demonstrated that the inhibition of XIAP by its endogenous antagonist Smac led to an improved survival and a reduction of glioma size when co-administered with the death ligand Apo2L/TRAIL *(Fulda et al., 2002a)*.

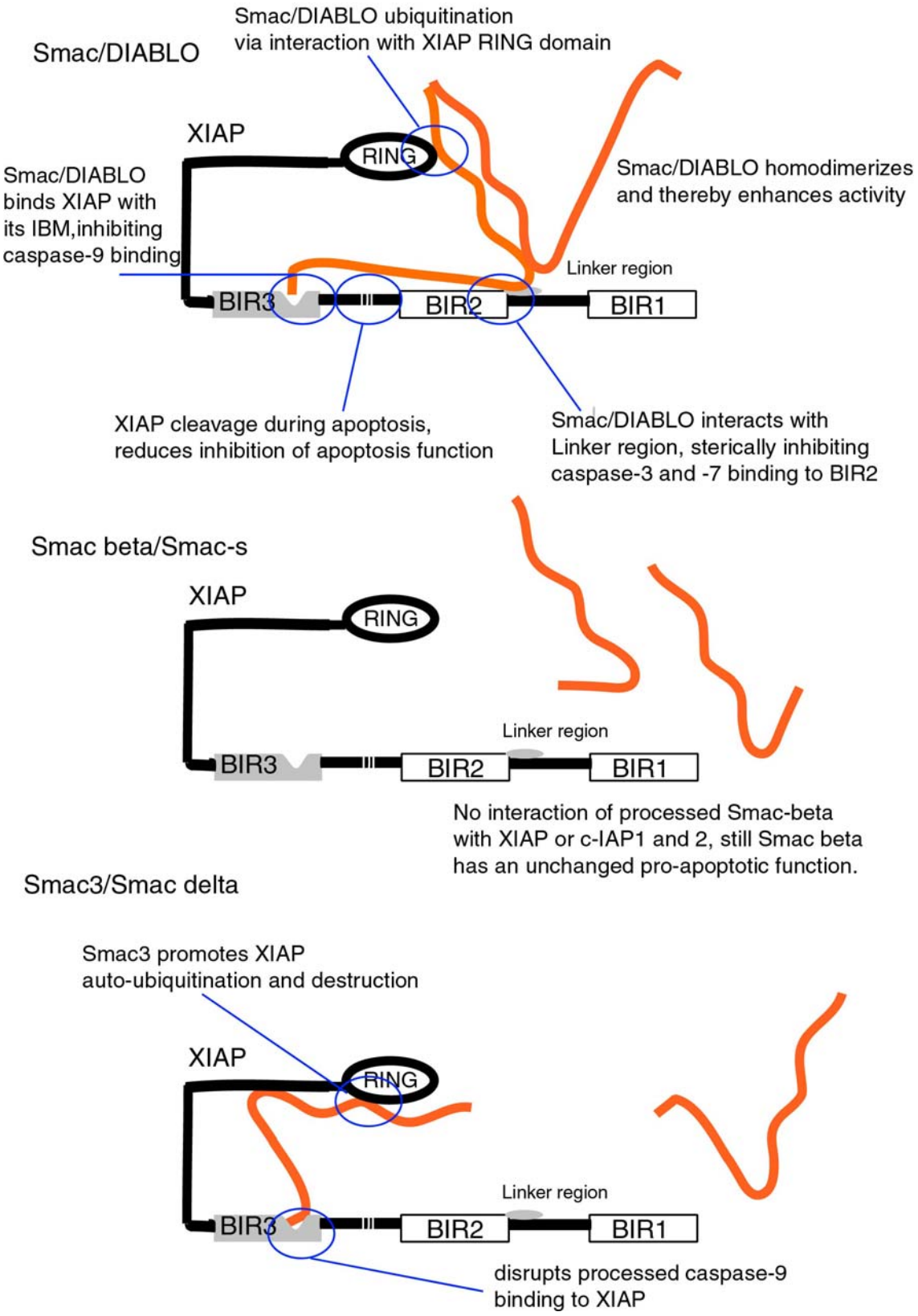

Figure 6. Summary of the interactions of Smac and its iso-types with XIAP

1.4 Aim and main questions of this work

The main aim of this study is to characterize the expression and to some extent the role of Smac and XIAP in astrocytic tumors of different grades of malignancy. With the technique of immunohistochemistry the activation of the two main pathways of apoptosis initiation, the death receptor pathway and the mitochondrial pathway are analyzed and discussed in conjunction with these two important regulators of apoptosis, pro-apoptotic Smac and anti-apoptotic XAIP. It is investigated whether these two antipodal proteins have a measurable association with the expression of cleaved caspases-8 and -9 and also on the rate of apoptosis in human astrocytic tumors in vivo (Figure 7).

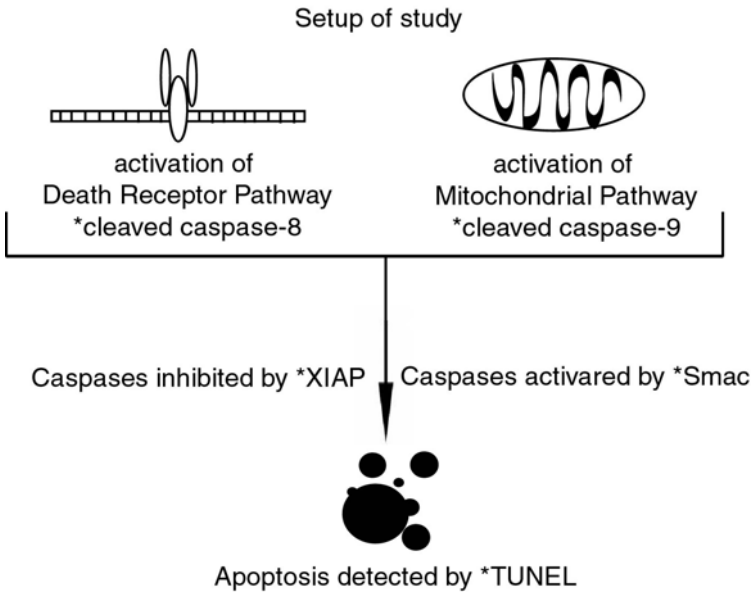

Figure 7: Simplified diagram of apoptosis regulation to demonstrate general setup of study, the analyzed proteins/assays are marked (*).

To determine if changes of the regulation of apoptosis exist alongside the increasing malignant phenotype from low to high grade, the expression of the four proteins and the TUNEL assay are assessed grade dependent in astrocytic tumors of various WHO grades.

The analysis of apoptosis is also performed in center regions and the infiltration zone of glioblastoma, to elucidate if differences in the regulation of apoptosis exist in the various regions of high grade glioma. As relapse glioblastomas

generally show a high degree of resistance towards genotoxic therapies, the expression of XIAP and Smac as well as cleaved caspase-8, -9 and the TUNEL assay were evaluated in primary glioblastomas and corresponding relapses to detect possible changes in the protein expression and apoptosis ratio.

Smac and XIAP are analyzed to a greater extent, as the outlook of future anti-XIAP therapies is evolving and grossly depends on the characterization of XIAP tissue expression in vivo with Smac constituting as a potential candidate for XIAP inhibition. Thus, for these proteins the analysis is additionally performed in normal brain tissue and statistical evaluation included correlations with patient age, patient sex and cellular density.

Finally, possible associations of the protein expressions of Smac and XIAP with survival were assessed, to detect if the endogenous protein expression of these two important regulators of apoptosis has a prognostic relevance for patient survival.

For the analysis of the above proteins on a large array of tumors, the TMA method was employed. To verify that the TMA method generates representative results in heterogeneous tumors such as the glioblastoma, the expression of the proliferation marker MIB-1, an established marker in astrocytic tumors, was analyzed.

The ratios of proliferation and apoptosis were also correlated to cellular density to evaluate if cellularity in astrocytoma depends more on the creation or on the degradation of tumor cells.

2 MATERIALS AND METHODS

2.1 Patient and Tissue selection

For the purpose of this study 194 brain tumor samples obtained from the tumor bank of the Institute of Brain Research (Neuropathology), University of Tuebingen, were investigated. The samples derived from patients who underwent surgical treatment in the neurosurgical departments of the University of Tuebingen and the Asklepios Clinic, Seesen from 1993 to 2003, consisting of 26 WHO grade I (pilocytic; 17 male (M), 9 female (F), median age 18 years) , 37 WHO grade II (29x fibrillary, 5x gemistocytic, 3x protoplasmatic; 20 M, 17 F, median age 38 years), 52 grade III astrocytomas (32 M, 20 F, median age 44 years) and 79 WHO grade IV glioblastomas (46 M, 33 F, median age 59 years). The group of glioblastoma consisted exclusively of primary glioblastomas, tumors developing with no obvious precursor, potentially with a differing genetic background to secondary glioblastoma deriving from lower grade gliomas and that only account for 5% of all glioblastomas *(Ohgaki and Kleihues, 2005)*. Additionally, 25 infiltration zones and 31 relapses of glioblastomas were included. 23 autopsy cases from a normal brain bank were also added.

The pathological diagnoses were confirmed by at least two neuropathologists. The histopathological typing and grading were done according to the WHO criteria for tumors of the nervous system *(Kleihues and Cavenee, 2000)*. The infiltration zone of glioblastomas was defined as an area adjacent to the tumor with a clearly reduced cellular density and no further signs of atypia like vessel abnormalities or necrosis.

One main limiting criterion of patient selection was the amount of tissue available. To assure a sufficient number of histological slides, a tissue thickness of at least 1 mm at the area of interest was demanded for the construction of the tissue microarray. While the tissue thickness after paraffin embedding was sometimes hard to judge, it was evident that many tissue samples of the brain bank did not meet this requirement and were thus not integrated into this study.

To have a reasonable time interval for survival analysis, no patients were selected with a primary operation after the 31st of December 2003. Starting at

this date all patients meeting the above requirements were consecutively included.

2.2 Preparation of the histological slides

2.2.1 Tissue fixation

Immediately after extraction the tissue samples were fixed in 4% formalin (pH 7.4). After fixation, all samples were dehydrated with a series of ethanol of increasing concentration (70%, 70%, 80%, 96%, 100%), followed by a mix of 100% ethanol and chloroform and finally undiluted chloroform. The tissues were then embedded in paraffin.

2.2.2 Tissue microarray (TMA) construction

For the immunohistochemical examination of the tissue samples the tissue microarray method was used. This technique has shown efficiency for high-throughput profiling of various tumor specimens *(Kononen et al., 1998)*. The method allows the simultaneous staining and examination of up to 1000 tissue samples. The tissue microarrays were constructed by extracting cylindrical tissue core biopsies from the above paraffin donor blocks followed by re-embedding these into a pre-punched hole on a single recipient (microarray) paraffin block at defined array coordinates. Using this technique, representative tissue sections were extracted and prepared as a tissue microarray. Prior to this tissue microarray construction a haematoxylin and eosin (H&E) stained slide of each block was analyzed in order to avoid inappropriate regions (e.g. necrosis, hemorrhage) on the TMA slides. The TMA machine (Beecher Instruments, Sun Prairie; USA; Figure 8) was used to extract a cylindrical core sample with a cross section diameter of 600 µm from the tissue donor block. Up to 108 samples were placed on a single recipient paraffin block (Figure 9). The tissue microarray blocks were then cut with a microtome (3µm thickness) and placed on SuperFrost Plus slides (Microm International, Walldorf, Germany).

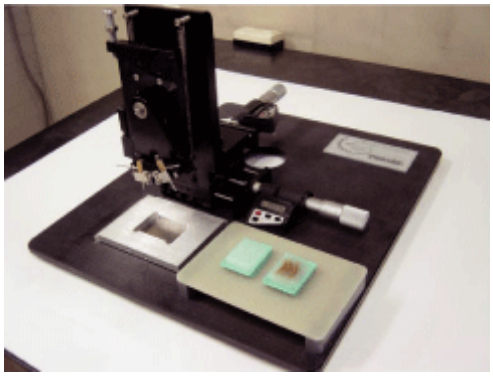

Figure 8. MTA-1 (manual tissue arrayer; Beecher Instruments; Image from Daiichi pure chemicals Co., Ltd).

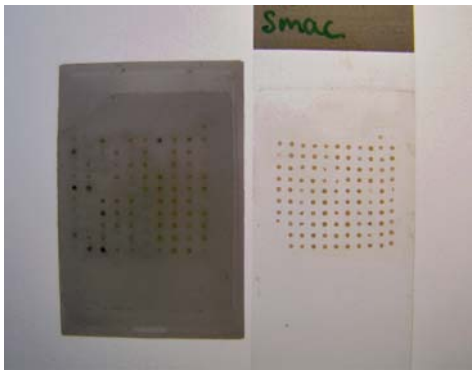

Figure 9. Tissue micro array paraffin block with 100 core biopsies and corresponding immunohistochemically stained slide (Smac).

2.2.3 Slide preparation for immunohistochemistry

For the preparation of immunohistochemical staining the slides were deparaffinized in chloroform for 30 minutes then rehydrated in descending series of ethanol (100%, 100%, 96%, 70%). Several unsuccessful staining experiments showed that the TMA slides dried up during the long incubation times of immunohistochemical protocols. Partly responsible for this was the low amount of tissue on each slide. A lack of tissue adherence of the working solutions and an extensive dispersal with a high evaporation on the glass surrounding the tissue was observed. This methodical problem was solved by carving a small dent around the array of tissue samples, thereby mechanically blocking the spreading of the fluid. A standard laboratory glass carver was used for this.

2.3 Immunohistochemistry

2.3.1 Outline of method

Today, immunohistochemical staining methods are widely used and represent one of many specific staining techniques. The central reagents of this method are immunoglobulins, usually of the IgG or IgM class. These classes can be further divided into sub-classes, e.g. IgG_1 and IgG_2. The primary antibodies are either polyclonal, i.e. generate from several B-lymphocytes and react with various epitopes of the antigen, or monoclonal, thus generated in one plasma-cell clone and are specific for an epitope of the protein which they were raised against. With the exception of a polyclonal antibody for cleaved caspase-9, only IgG antibodies of the IgG_1 sub-class were used in the final analysis of this study.

The aim of immunohistochemistry is to then produce a visible stain at the sight of antibody binding. Several techniques for such reactions exist, the most commonly used being based upon the strept(avidin)-biotin method. The following paragraph concentrates on this technique, as it was also used for the immunohistochemical antigen detection of this study.

After the binding of the specific antibody (primary antibody) an incubation with a second antibody (secondary or link antibody) follows. As the F_c fragment is generally constant within one species (mouse, goat etc.) it is possible to produce specific antibodies against this region of the primary antibody. The secondary antibody meets this requirement and is additionally biotinylated at its constant region. This bound biotin has a very high affinity for streptavidin ($K_m=10^{-15}$ M) and avidin. This step is followed by an incubation either with a Strept(Avidin)-Biotin-Enzyme-Complex (ABC method) or as in the presented study with enzyme labeled streptavidin (so called LSAB method for Labeled StreptAvidin-Biotin method) which is rapidly bound to the biotinylated antibodies due to the high affinity of biotin and streptavidin. An often used enzyme for this labeling is the horseradish peroxidase (HRP), as it is relatively cheap, it can be covalently-bonded to streptavidin without losing its activity, it is soluble in water and almost no endogen enzyme activity exists. Thus, the specific binding of the

primary antibody leads (via two intermediate steps) to a high concentration of an enzyme with peroxidase activity in the proximity of the sought-after protein. In the presence of an electron donor the peroxidase causes the catalysis of H_2O_2, while the oxidation of the electron donor itself leads to precipitation and conversion to a colored compound. In the used protocols the chromogen diaminobenzidine (DAB) functioned as the electron donor. It generates a brown reaction product that is insoluble in organic solvents. Its oxidation is then followed by a polymerization, thereby further increasing its luminosity *(Boenisch et al., 2003)*.

2.3.2.1 Selection of Smac and XIAP directed antibodies

For both antigens two different antibodies were tested for possible application in immunohistochemistry. All four antibodies were specific against epitopes of Smac and XIAP and were tested on all the above tissues but only two of them (one for XIAP and one for Smac) were usable for immunohistochemical assays. The implementation of a polyclonal anti-human XIAP antibody from immunized rabbits raised against amino acids 244 to 263 of human XIAP (R&D Systems, Minneapolis, USA) was not possible, as the antibody did not produce any sort of staining. For Smac the establishment of a polyclonal antibody against recombinant mouse Smac amino acids 55 to 237 also did not yield satisfactory results (Alexis Biochemicals, Lausen, Switzerland), again it was hardly possible to detect any staining. The two functioning antibodies are characterized below.

2.3.2.2 Smac

The final evaluation of Smac expression was performed with an IgG_1 monoclonal mouse anti-Smac antibody (clone 17-1-87; Zymed Laboratories, South San Francisco, USA; dilution 1:100). Full length human Smac was used as immunogen.

2.3.2.3 Pretreatment and immunostaining (Smac)

For antigen retrieval the slides were boiled in citrate buffer (pH 6.0) in a microwave oven at maximum power (800 Watt) for 8 min, then allowed to cool down for 10 min. After washing with PBS buffer the endogenous peroxidase was eliminated by incubation in 0.05% hydrogen peroxide in 100% methanol for 15 min at room temperature. After blocking endogenous biotin (see below) the slides were washed in PBS again followed by treatment with TNB buffer (0.1 mol/L TRIS-HCl, pH 7.5, 0.15 mol/L NaCl, 0.5% blocking reagent) to reduce background staining. The slides were incubated overnight at 4ºC with the antibody for Smac. They were then incubated with biotinylated secondary antibodies (anti-mouse antibody, 1:400, DakoCytomation, Carpinteria, USA) for 30 min at room temperature. The first treatment with streptavidin-peroxidase for 30 min at room temperature was followed by an enhancement step with biotinylated tyramide as an additional signal enhancement (see below). A second streptavidin-peroxidase treatment for 30 min followed. After each incubation step the slides were washed in PBS twice for 10 min on a laboratory shaker. For visualization the slides were incubated with DAB/H_2O_2 for 30 s, counterstaining was performed with haematoxylin.

Blocking endogenous biotin

Both the biotin of the exogenous biotinylated secondary antibodies and endogenous biotin (vitamin B7/ vitamin H) function as binding partners for avidin (about 0.05% of egg albumen, used in the standard ABC method) and of streptavidin (derived from Streptomyces avidinii, used here for Smac detection). Avidin and streptavidin have four binding sites for biotin. Endogenous biotin is a coenzyme in many reactions and has been found in high concentrations in liver, kidney and lymphatic tissue. Lower concentrations were also found in the CNS and it was described to produce non-specific staining in avidin-biotin based visualization systems *(Hsu and Raine, 1984)*. In this study endogenous biotin was blocked using the Biotin Blocking System (DakoCytomation, Carpinteria, USA). After antigen retrieval in the microwave the slides were incubated with 0.01% avidin for 15 min to bind endogenous biotin. The additional three binding

sites of avidin were then blocked in a following incubation with 0.01% biotin for 15 min. After washing the slides thoroughly the protocol proceeded with the incubation with TNB buffer and Smac primary antibody.

Signal enhancement with biotinylated tyramide

This method which is commonly called CSA (Catalysed Signal Amplification) functions by the introduction of the biotinylated phenol tyramide as an additional substrate for the avidin-bound peroxidase. When the substrate is added after the incubation with HRP labeled streptavidin the tyramide is transformed into an insoluble form and precipitates close-by. Its bound biotin also accumulates in the proximity. This additional load of biotin functions as a binding partner for a second incubation with HRP labeled streptavidin (LSAB method, see above), thereby leading to an amplification of the original signal. In some cases an increase of sensitivity by a factor of 100 has been described *(Sanno et al., 1996)*, and the method has displayed advantages for the detection of sparse proteins *(von Wasielewski et al., 1997)*. In this study the Renaissance TSA Biotin System (Perkin Elmer, Boston, MA) was used with an biotinylated tyramide incubation time of 7 min.

2.3.2.4 XIAP

XIAP immunohistochemistry was performed by applying a mouse monoclonal IgG_1 antibody raised against aa 268-426 of recombinant XIAP peptide in a dilution of 1:100 (clone 48, BD Biosciences Pharmingen, San Diego, USA).

2.3.2.5 Pretreatment and immunostaining (XIAP)

For XIAP, immunohistochemical tissue labeling was performed by using the Benchmark immunohistochemistry system (Ventana, Strasbourg, France). Endogenous peroxidase of the tissue sections was blocked with 3% H_2O_2 in methanol for 14 min. A cell conditioning pretreatment was performed for 32 min. The primary antibody was incubated for 32 min. An avidin and a biotin blocker were applied to the samples for 4 minutes respectively followed by an 8 min incubation with one drop of I-View-Biotin Ig (Ventana). For DAB visualization, the sections were incubated with one drop of I-View SA-HRP for 8 min and then

with DAB/H_2O_2 for an additional 8 min. The sections were finally incubated with a copper enhancer (Ventana, Strasbourg, France) for 4 min, washed, counterstained with haematoxylin and mounted.

2.3.2.6 Immunohistochemical detection of cleaved caspase-8, and -9

To detect the levels of activated caspases, antibodies specifically detecting the cleaved forms of caspase-8 (ccasp-8) and cleaved caspase-9 (ccasp-9) were used.

For ccasp-8 a monoclonal mouse antibody was used which does not cross react with full-length caspase-8 (Calbiochem, San Diego, USA, monoclonal mouse IgG_1, dilution 1:75).

For ccasp-9 immunohistochemical staining was performed with a polyclonal rabbit antibody which does not recognize full-length caspase-9 or any other caspase (BIOZOL, Eching, Germany, dilution 1:10).

The immunohistochemical tissue labeling for both caspase antigens was performed as described for XIAP.

2.3.2.7 Isotype and negative controls

As a isotype control, every slide was also treated with replacement of primary antibody with a monoclonal IgG_1 antibody for an aspergillus niger surface protein (DakoCytomation, Carpinteria, USA), a protein structure not expressed in mammalian tissue in the same concentration as the primary antibody. A positive staining suggests an unspecific binding of the IgG_1 antibody to an unknown epitope in the tissue. For XIAP one tumor exhibited unspecific staining and for Smac three tumors. The tissues were excluded from further evaluation.

For the polyclonal antibody ccasp-9 the negative control was performed by replacing the primary antibody with PBS.

2.3.2.8 Assessment of proliferation activity (MIB-1 staining)

MIB-1 is a monoclonal antibody against Ki-67, a non-histone nuclear matrix protein that has an as yet undefined role in the proliferative process *(Gerdes et*

al., 1991). It labels cells in the G1, S and G2/M phases of the proliferative cell cycle (DakoCytomation, Carpinteria, USA).

2.4 TUNEL staining

As one of the hallmarks of programmed cell death, endonuclease-mediated cleavage of DNA results in extensive DNA breakage in the nuclei of cells undergoing apoptosis. The 3' termini of the strand breaks can be detected by attaching specifically labeled nucleotides. This reaction is catalysed by exogenous terminal deoxynucleotidyl transferase (TdT), and the breaks are labeled with fluorochrome-tagged deoxynucleotides (mostly dUTP). The method has been called TUNEL, short for TdT-mediated dUTP-biotin nick end-labeling *(Gold et al., 1993)* and has found wide application in the detection of spontaneous apoptosis.

A commercially available kit was used (Roche, Mannheim, Germany) and was performed according to the manufacturer's instructions.

2.5 Light microscopy and counting

To quantify the reaction of Smac, XIAP, ccasp-8 and MIB-1 antibodies 200 cells per sample were counted in a predefined fashion; a clear granular or cytoplasmatic stain was counted positive. For MIB-1, a nuclear staining was counted positive. If less than 200 cells were present in the tissue selection (common in normal brain) all available cells were counted. In the tumor and in grey and white matter of normal brain intra-luminal, endothelial, parenchymal inflammatory and neuronal cells were excluded. Tumors expressing less than 1% positive cells were considered as negative for the purpose of protein expression for the above antibodies. Due to the low levels of TUNEL and ccasp-9 staining all neoplastic cells within a predefined area of 0.25 mm² were evaluated. The ratios of positive cells were calculated as stained cells divided by neoplastic cells in the area. Only TUNEL positive cells with morphological signs of apoptosis were counted as positive. The cell density was surveyed in the infiltration zone and corresponding glioblastoma center in a counting grid

and evaluated as cell count per calibrated area (mm²). Evaluation of the immunohistochemical staining and photographic documentation was performed using an Olympus BX50 light microscope.

2.6 Acquisition of survival data

The acquisition of personal data (age, sex and survival time after first operation) was performed in cooperation with the Department of General Neurology and the Department of Neurosurgery, University of Tuebingen. While age, sex and very few survival data were taken directly from the patient files the majority of cases was acquired in personal contact with the patients or the bereaved family members. The 15th of December 2005 was fixed as the end of survival observation (last follow up). Astrocytoma patients could be followed up within a period of up to 12.6 years. Within this time frame a total of 102 patients died after a mean survival of 22.2 months (range 1-151) whereas 78 were still alive with a mean follow up of 44.4 months (range 1-136). 54 of the 102 deceased had been diagnosed with grade IV tumors, 31 with grade III, 16 with grade II and only 1 patient with grade I. 14 patients were lost trace of immediately after operation, so this population could not be integrated into the Kaplan-Meier survival analysis.

2.7 Statistical analysis

In order to stabilize variance, fractions of positive cells were transformed by taking the arcsine of the square-root. The means were compared by a one-way analysis of variance (ANOVA) and for the post-hoc testing Tukey-Kramer's HSD test was used with a global significance level of 5%. For significant differences individual p-values were calculated. For individual pair wise comparisons a one sample t-test was used. The back-transformed means are given together with their 95% confidence intervals (CI), significant differences are illustrated (* $0.01<p\leq0.05$, ** $0.0001<p\leq0.01$, *** $p\leq0.0001$). For assessing the correlation between age and antigen expression and between TUNEL and antigen expression Spearman's rho was used. To describe patient survival, a univariate survival analysis using product limit (Kaplan-Meier) life table was used for grade

II, III and IV gliomas. The analysis was not performed for grade I because of only one observed case with a primary endpoint (death). Samples were stratified by protein expression into three groups of approximately equal size (tertiles) to perform Kaplan-Meier analysis (high, mid and low expression). In order to compare these survival curves the Wilcoxon test for censored data was used. Exponential distributions were fitted to the individual survival curves by maximum likelihood and tested for differences by the likelihood ratio criterion. For adjustment of the p-values due to multiple testing the method of Bonferroni-Holm was used. JMP IN (www.JMP.com) was used for statistical analysis.

3 RESULTS

3.1 General findings

Cellular density

The evaluation of tumor cells per mm² (c/mm²) yielded an increasing density from the lower WHO grade tumors to the tumors of higher grades (Figure 10a). While WHO grade I (pilocytic) and grade II were at approximately similar levels, i.e. 2400 c/mm² (CI 2000 to 2800) and 2000 c/mm² (CI 1700 to 2300) respectively, cellularity increased to 3200 c/mm² (CI 2800 to 3600) in anaplastic astrocytoma ($p=0.0053$ and $p<0.0001$, respectively). Cellularity was highest in glioblastoma with 4500 c/mm² (CI 4000 to 4900) and this was highly significant different to all other grades ($p<0.0001$). Even in WHO grade I and II tumors the cellularity was still higher than in both grey (900 c/mm², CI 800 to 1000) and white matter (1400 c/mm², CI 1200 to 1500) of the frontal lobe of human normal brain. In normal brain the cell density was significantly lower in grey matter as compared to white matter (mean ratio 1.55 (CI 1.37 to 1.7) $p<0.0001$, Figure 10b). These results are in line with histopathological observations and reflect the reliability of the tissue selection.

The evaluation of cellular density in the infiltration zone showed a highly significant decrease of cellularity with a mean ratio of 2.1 (CI 1.68 to 2.64) from glioblastoma to corresponding infiltration zone ($p<0.0001$; Figure 10c). The geometric mean cellularity in the infiltration zone of 1900 c/mm² (CI 1600 to 2300) was still considerably higher than in the normal brain.

In the relapse cases a significant reduction of cellular density from the primary tumor to the recurrent gliomas was observed (mean ratio by 1.49 (CI 1.05 to 2.00); $p=0.013$; Figure 10d).

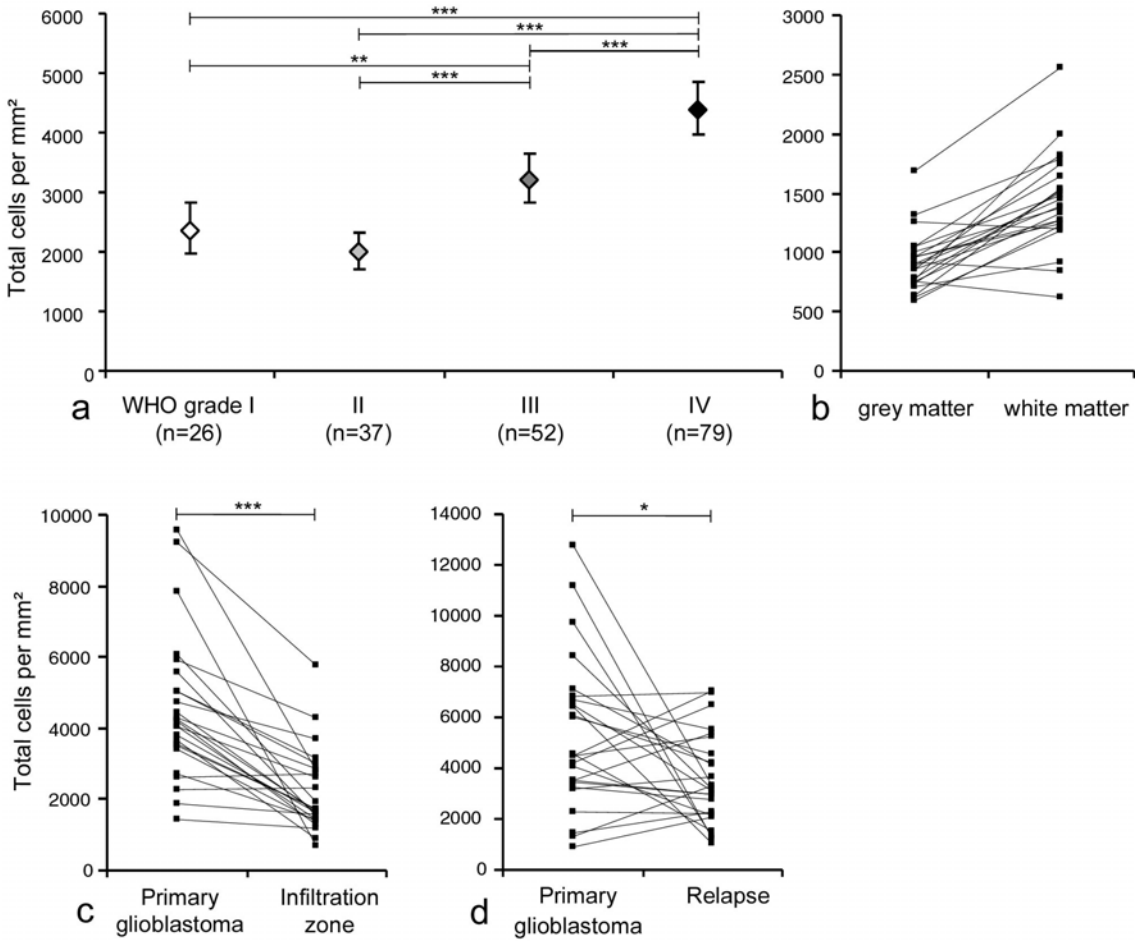

Figure 10. General findings. (a) Illustration of cellular density in astrocytoma tissue grade I-IV. (b-d) Depiction of a matched pairs analysis of cellular density in normal brain (b), in the infiltration zone of glioblastomas (c) and in relapses of glioblastomas (d). Corresponding tissue pairs are connected with a line.

MIB-1 staining

Even though the staining of the proliferation marker MIB-1 is an immunohistochemical procedure it is presented and later discussed as a part of the general findings. This is due to the widespread application and general acceptance of this procedure. The evaluation of MIB-1 showed a clear increase in high grade astrocytomas. While the difference between grade I and II was not significant, highly significant differences could be detected between all other grades ($p<0.0001$; Figure 11). The mean expression in pilocytic astrocytoma was 0.2% (CI 0-0.6%), 0.5% (CI 0.16-1.1) in grade II, 3.3% (CI 2.3-4.4%) in grade III and as high as 9.5% (CI 8.2-10.9%) in grade IV glioblastoma. Due to

technical problems of the TMA staining not all tissues were evaluable (note reduced numbers of tissues in Figure 11).

The correlation of MIB-1 staining with cellular density displayed that while there was a highly significant association in the entire group of astrocytomas (grade I-IV; $p<0.0001$, data not shown), only grade IV demonstrated a significant association in the grade dependent analysis ($p=0.015$). For grade III the correlation was nearly significant ($p=0.056$) whereas grade I and II exhibited no correlation of proliferation activity and cellular density ($p=0.61$ and $p=0.6$ respectively, data not shown).

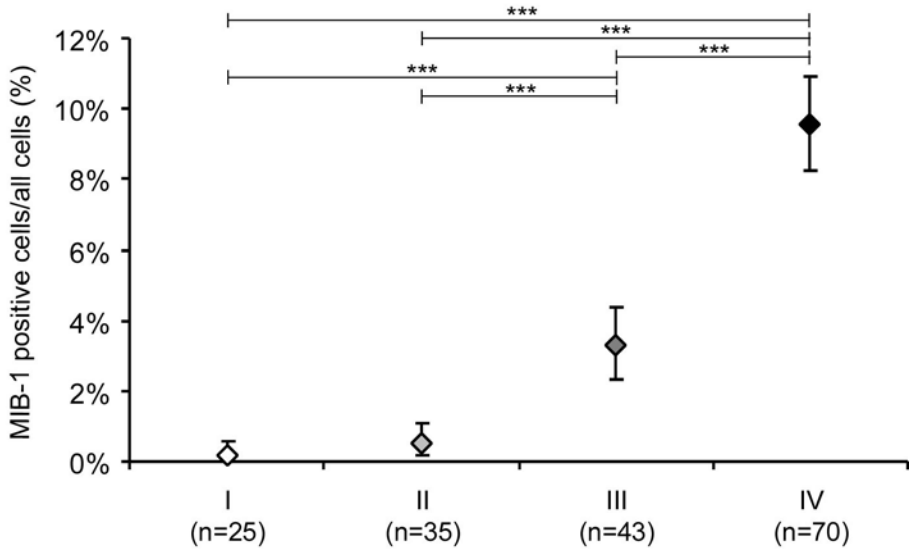

Figure 11. MIB-1 expression in glioma grade I to IV (mean and 95% CI).

3.2 Immunohistochemistry

3.2.1 XIAP expression in astrocytoma and normal brain

The levels of XIAP protein expression in neoplastic cells showed a steady increase from grade I to grade IV tumors with significant differences from grade I to III, I to IV and II to IV ($p=0.0053$, $p<0.0001$ and $p=0.0031$ respectively) (Figure 12a-d and Figure 13a). Only 3.2% (CI 0.5-8.1%) of WHO grade I (pilocytic) tumor cells were positive, rising to 8.2% (CI 4.0-13.7%) in grade II and 13.1% (CI 8.4-18.7%) in anaplastic astrocytoma grade III and to 19.2% (CI 14.6-24.3%) positive cells in the WHO IV glioblastoma. In grade I 36% of the

tumors were negative for XIAP, in grade II 30%, in grade III 12% while in grade IV only 10% of the tumors were not expressing XIAP. The XIAP expression levels of glial cells in the normal brain were lower than in WHO grade I tumors with 0.8% (CI 0.06-2.5%) in the grey and 2.3% (CI 0.7-4.8%) in the white matter (Figure 12e, f and Figure 13b). Neuronal cells in the grey matter of normal brain and infiltration zone of glioblastoma did not express XIAP (Figure 12e).

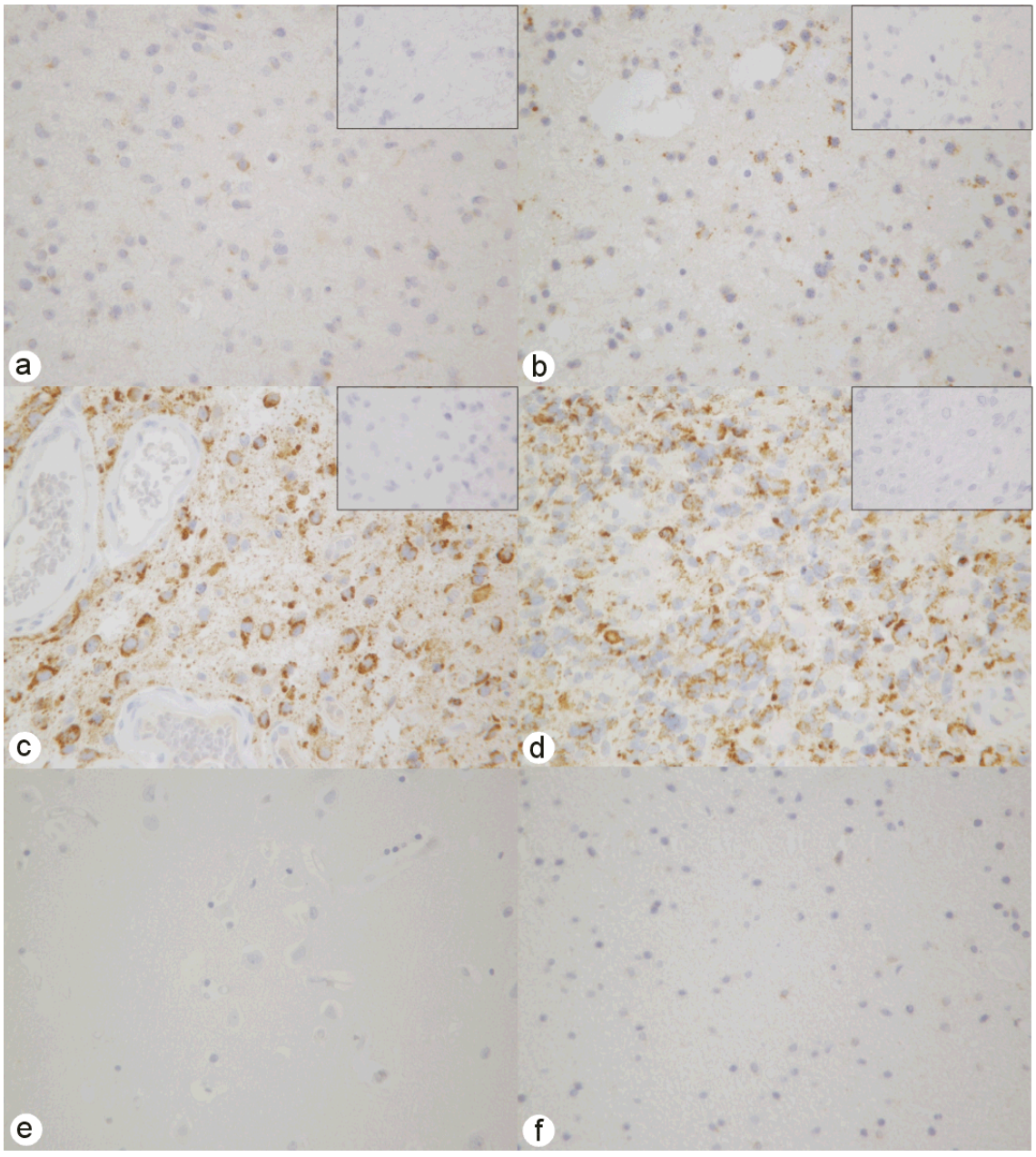

Figure 12: Representative micrographs of XIAP immunohistochemistry in tumor tissue and normal brain (immunostaining with diaminobenzidine, nuclear

counterstain with haematoxylin). XIAP expression levels continuously increase from WHO grade I pilocytic astrocytoma (a) over WHO grade II (b) and III (c) gliomas to the glioblastoma WHO grade IV (d). In contrast, normal grey (e) and white (f) matter did not show relevant XIAP immunoreactivity. Insets a-d IgG_1-isotype controls. Original magnification 400x for all micrographs.

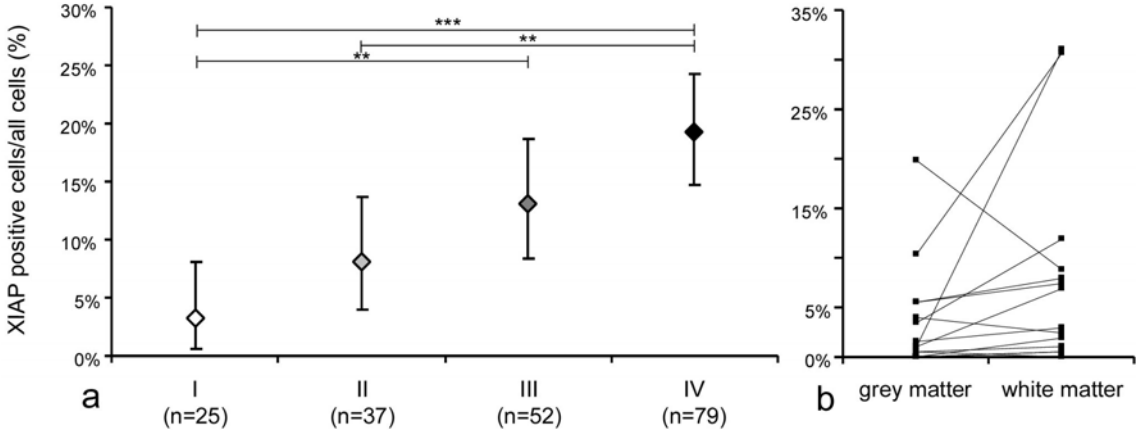

Figure 13. XIAP immunostaining in the tumors and the normal brain measured as percentage of immunoreactive cells of all cells. At least 200 cells per tissue sample were counted and the percentage of positive cells was assessed. (a) XIAP expression in glioma grade I to IV demonstrating a steady increase from low to high grade gliomas (mean and 95% CI). (b) XIAP expression in the normal brain showed no significant difference of grey and white matter.

3.2.2 Smac expression in astrocytoma and normal brain

Because of technical problems of the TMA slide staining, 3 grade I tumors and 2 grade III tumors could not be integrated into the Smac analysis while 2 grade III tumors and one glioblastoma exhibited unspecific staining and were also omitted. For Smac immunohistochemical staining Grade III tumors showed the lowest expression with high significance to grade II ($p=0.0001$) and also to grade IV ($p<0.0001$) (Figure 14a-d and Figure 15a). WHO grade IV tumors showed the highest ratio among all neoplasms and were also significant to grade I ($p=0.0012$). The percentage values were lowest in WHO grade III with 15.8% (CI 12.4-19.6%) followed by 22.2% (CI 16.5-28.4%) in grade I and 28.2% (CI 23.3-33.4%) in grade II tumors and were highest in WHO IV glioblastomas

with 34.3% (CI 30.7-38.1%) Smac positive cells. None of the tumors grade I to IV was negative for Smac protein. Smac levels of glial cells in normal brain were lower than in all the tumors, 12.5% (CI 8.3-17.3%) in the grey and 7.1% (CI 4-11%) in the white matter. According to the one-sample t-test, expression was significantly higher in the grey matter compared to the white matter (p=0.035, mean difference 0.8%; CI 0.005-3.1%, Figure 14e, f and Figure 15b). Neuronal cells were generally strongly positive for Smac when seen in normal grey matter or in the infiltration zone of glioblastoma (Figure 14e).

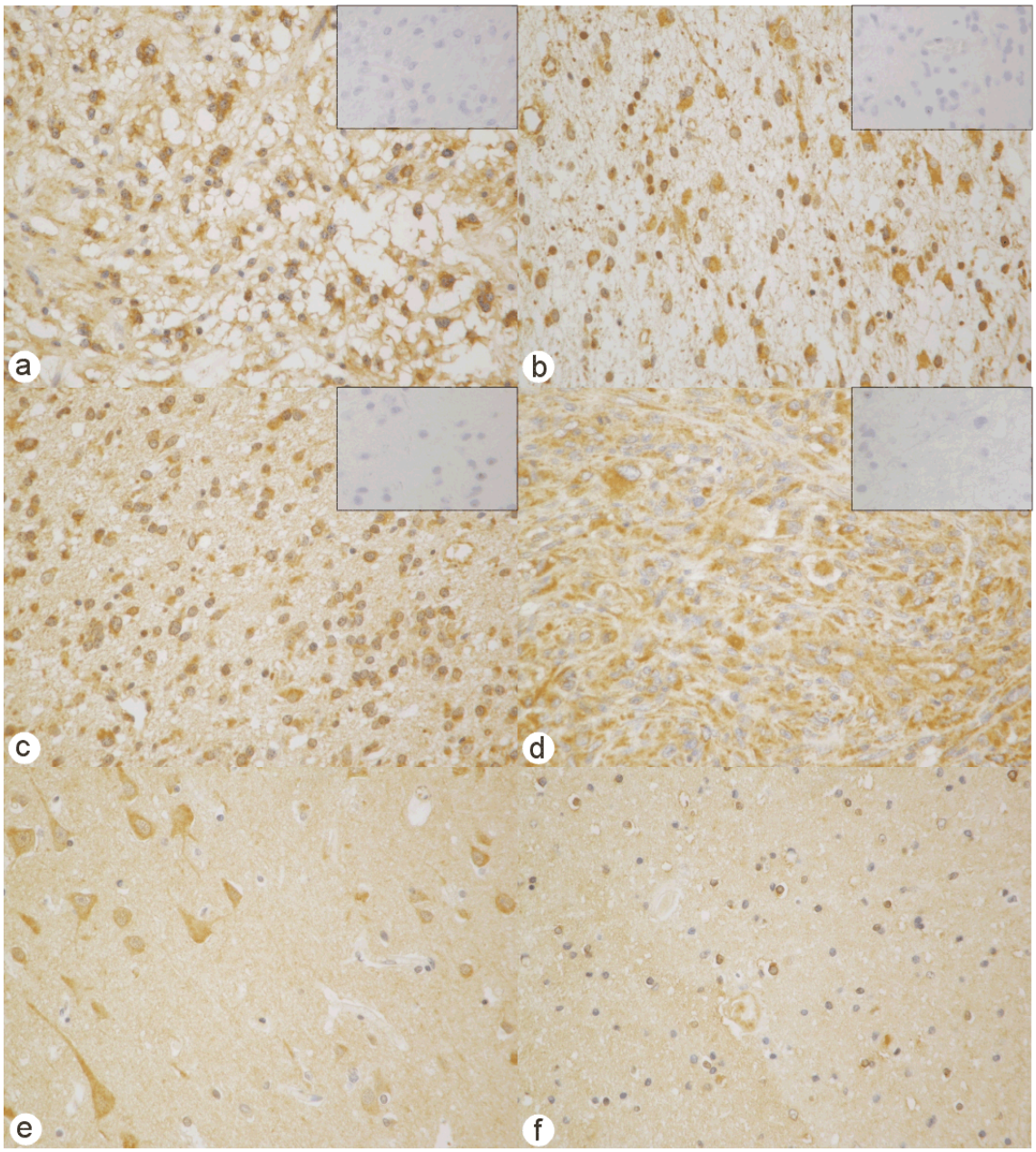

Figure 14. Representative micrographs of Smac immunohistochemistry in tumor tissue and normal brain (immunostaining with diaminobenzidine, nuclear counterstain with haematoxylin). The steady increase of SMAC expression from WHO grade I (a) and II (b) to WHO grade IV (d) tumors was interrupted by the lower SMAC levels in WHO grade III anaplastic astrocytomas (c). SMAC expression was significantly higher in normal grey (e) compared to white (f) matter. Neurons in the grey matter were generally strongly positive for Smac (e, large cells on the left). Insets a-d IgG_1-isotype controls. Original magnification 400x for all micrographs.

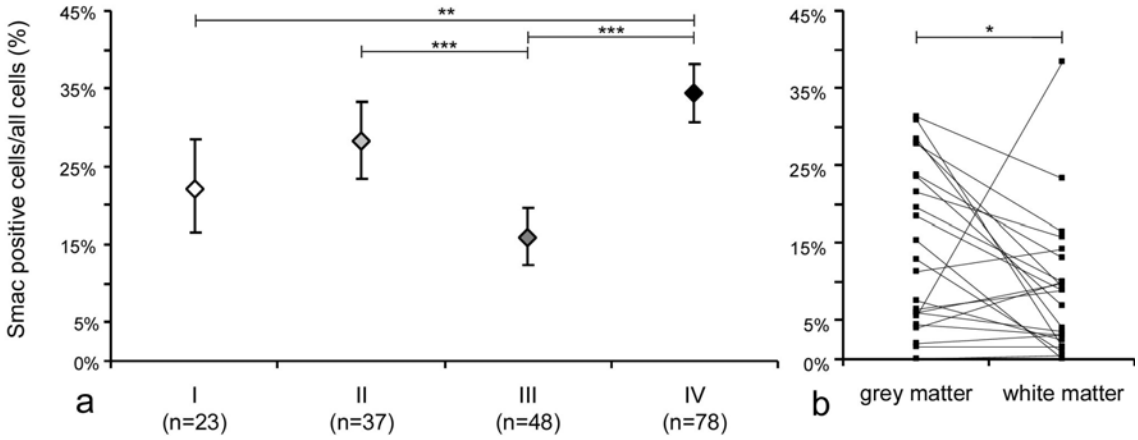

Figure 15. (a) Smac expression in glioma grade I to IV (mean and 95% CI). (b) Smac was expressed significantly lower in glial cells of the white matter compared to grey matter.

3.2.3 XIAP and Smac in the infiltration zone

Expression analysis of XIAP in the infiltration zone of glioblastoma revealed high levels with no significant differences to the corresponding tumor tissue in a matched pairs analysis (infiltration: 19.8% CI 10.8-30.6%, tumor 21.2% CI 11.9-32.3%; Figure 16a, b and Figure 17a). A strong correlation of individual XIAP levels in the infiltration zone and corresponding tumor was detected, i.e. individuals with high levels in the tumor showed high expression in the infiltration zone and vice versa (p=0.0002) (Figure 17b). The individual XIAP expressions in white and in grey matter of the normal brain also correlated highly (p=0.0003, data not shown).

In contrast, Smac is expressed significantly higher in tumor tissue than in the infiltration zone (infiltration: 23.8% CI 18.4-29.6%, tumor: 35.5% CI 29.4-42.0%; Figure 16 c, d and Figure 17c) and no correlation of expression in these two regions was detectable (Figure 17d). The same was the case in normal brain, where no correlation of individual white and grey matter was observed (data not shown).

Akin to the distribution of XIAP, the analysis of the regional expression of Ki-67 (detected by MIB-1) demonstrated a strong correlation of the infiltration zone and the tumor center (p=0.0026; data not shown).

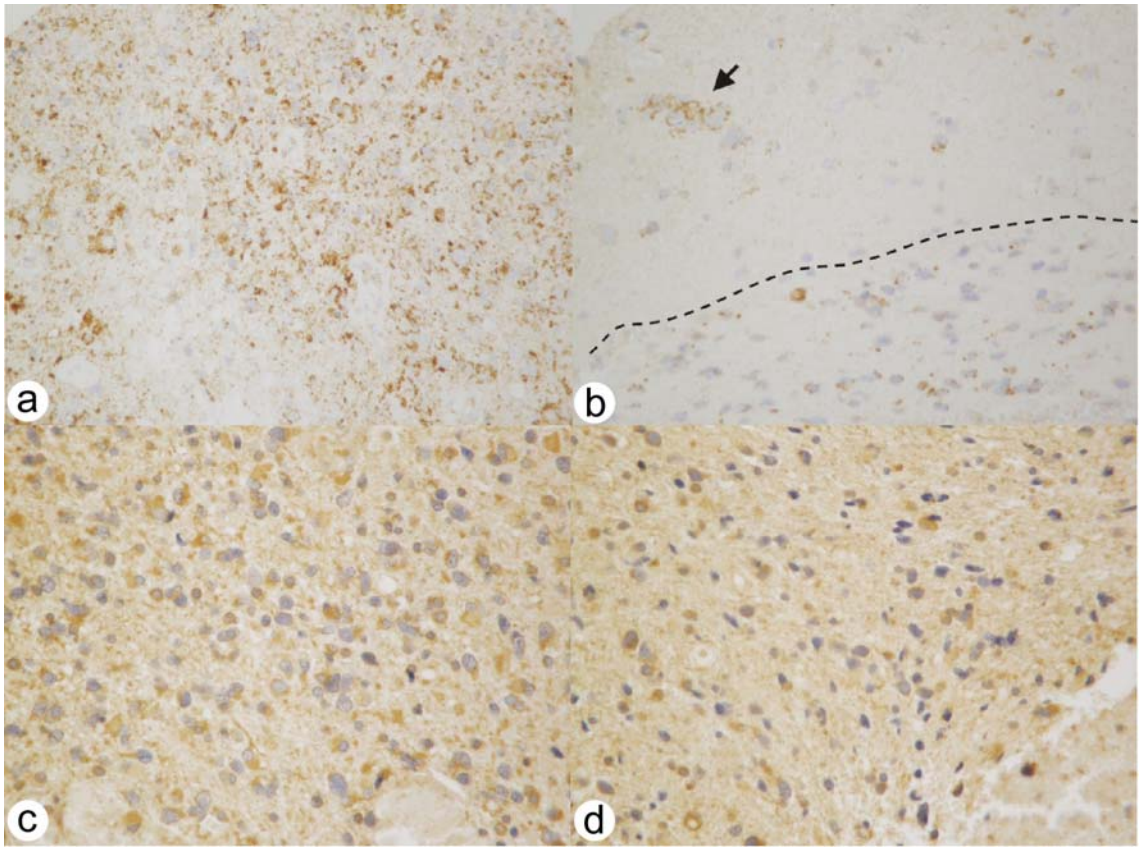

Figure 16. Representative micrographs of XIAP and Smac in the infiltration zone of glioblastoma. While the comparisons of tumor tissue (a) and corresponding infiltration zones (b) did not show significant differences for XIAP, Smac expression was significantly higher in the tumor (c) than in the infiltration zones (d). Tumor cell nests (b, arrow) within normal appearing CNS tissue (b, separated from the tumor by a dashed line) frequently showed strong XIAP expression.

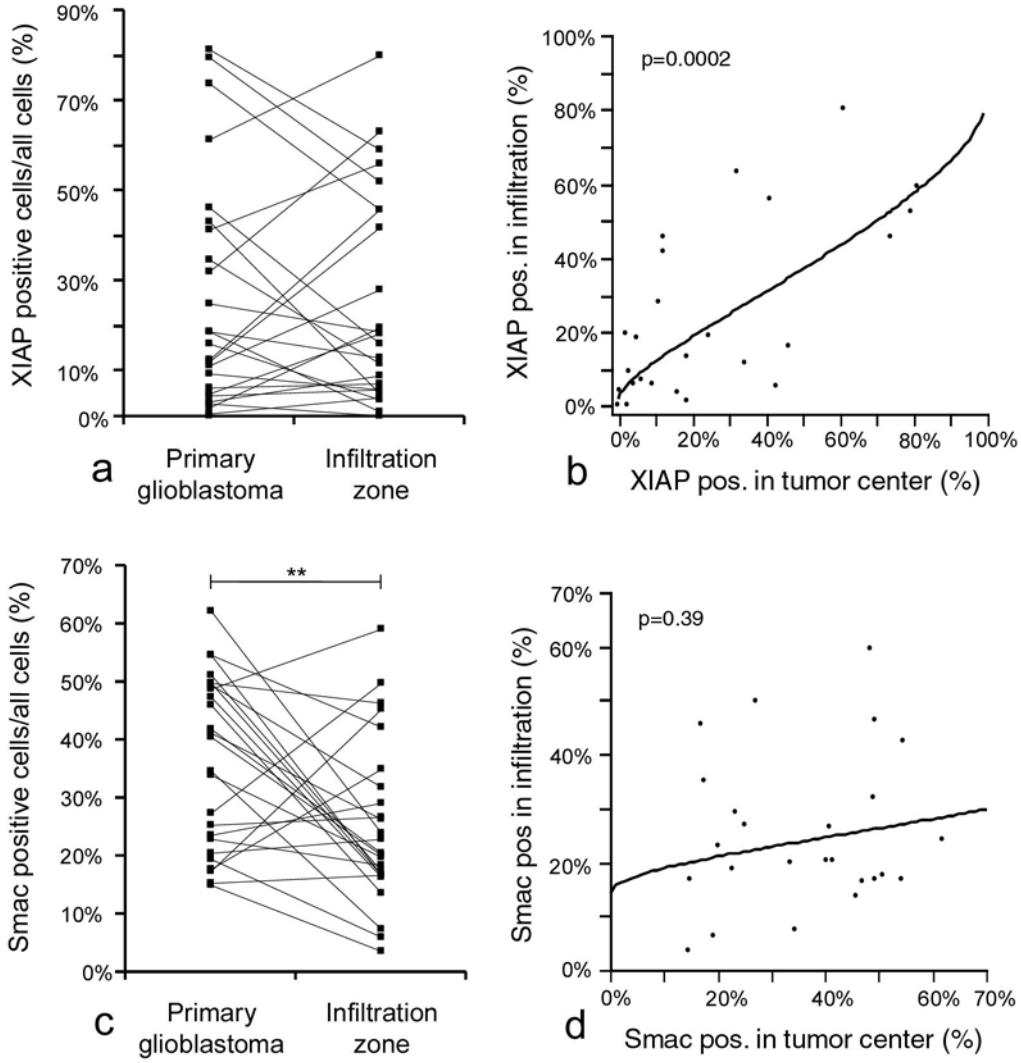

Figure 17. XIAP and Smac expression in the infiltration zone of glioblastomas. (a) Comparison of XIAP protein in glioblastoma and adjacent infiltration zone. Depiction of a matched pairs analysis, corresponding tissue pairs are connected with a line. While XIAP levels in individual tumors change, no significant trend of the protein expression was detectable. (b) Bivariate fit of XIAP in tumor and infiltration zone detecting a strong correlation of protein levels. (c) The analysis of Smac revealed a significant reduction of Smac in the infiltration zone (p=0.005, mean difference 1.65% (CI 0.18-4.57 %). Mean expression of Smac decreased from 35.5% (CI 29.4-42.0%) to 23.8% (CI 18.4-29.6%) in the infiltration zone. (d) Bivariate fit of Smac in tumor and infiltration detecting no correlation of protein levels.

3.2.4 XIAP and Smac in relapse glioblastoma

Although mean XIAP expression levels in recurrent glioblastomas were low (9.8%; CI 5.2-15.6%), no significant differences were found in the analysis of the tumors and individual relapses (p=0.86; data not shown). Smac expression was reduced in the relapses with a mean expression of 30.9% (CI 24.9-37.4%) compared to 39.8% (CI 33.3-46.5%) positive tumor cells in corresponding primary tumors, though this difference did not quite reach significance (p=0.054; Figure 18).

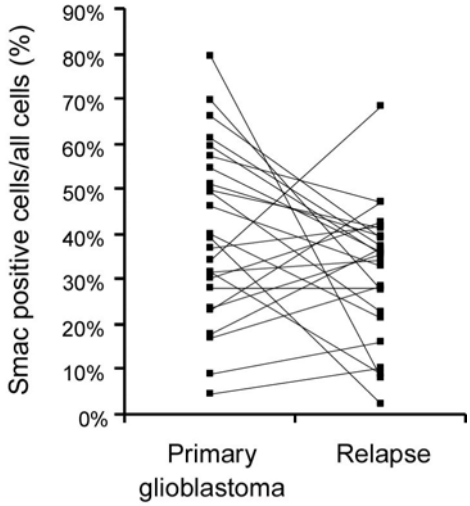

Figure 18. Illustration of Smac expression in primary glioblastoma and relapse glioma. Depiction of a matched pairs analysis, corresponding tissue pairs are connected with a line.

3.2.5 Correlation of antigen expression with age, sex, cellular density and proliferation

Age

In an analysis of patient age and protein levels a significant correlation of age and XIAP expression was detected exclusively in glioblastoma, while none of the other tumor grades or normal brain showed any association (Spearman's rho correlation coefficient 0.26, p=0.02, Figure 19). For example, XIAP is expressed nearly twice as highly in patients 60 years and over, with 25.9% (CI 18.1-34.6%) positive cells compared to 13.6% (CI 7.9-20.5%) in patients under

60 years of age (data not shown). Smac showed no such interrelationship (data not shown).

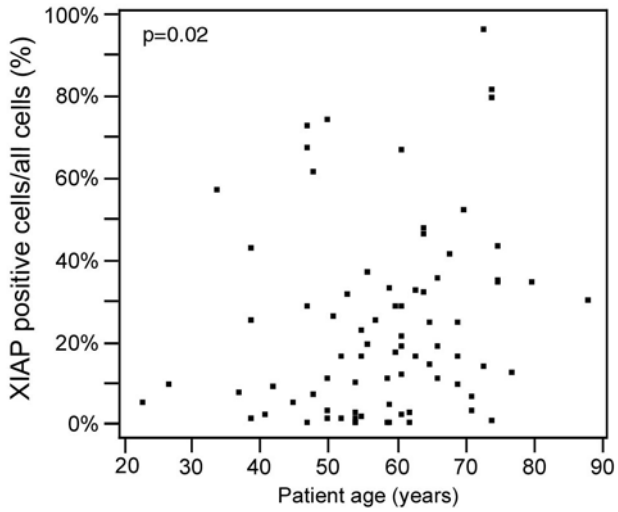

Figure 19. Illustration of XIAP expression in glioblastoma in association with patient age. For statistical analysis Spearman's rho was performed.

Sex

No sex specific differences of XIAP or Smac expression were detectable in any of the tumors and normal brain (data not shown).

Cellular density

The evaluation of Smac and XIAP expression in association with cellular density showed a significant correlation of Smac in glioblastoma with low levels of Smac when cellular density is high (p=0.003; Figure 20). This was neither observed for Smac in tumors of grade I to III nor for XIAP in any of the grades (data not shown).

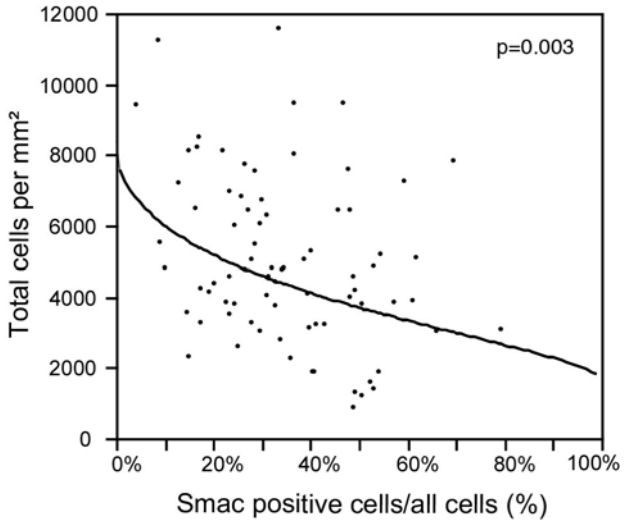

Figure 20. Bivariate fit of Smac in glioblastoma and cellular density demonstrating a strong negative correlation.

Proliferation

No correlation of XIAP or Smac protein with the expression of MIB-1 was observed in any of the tumor grades (data not shown).

3.2.6 Correlation of XIAP and Smac antigen expression

No correlation of XIAP and Smac protein was detectable in any of the tumor grades WHO I-IV (data not shown).

3.2.7 Expression of cleaved caspase-8 and -9 and correlation with XIAP and Smac expression

The evaluation of the active forms of caspases-8 and -9 in astrocytoma tissue demonstrated diverse results. For both antibodies several tissues were not evaluable due to technical problems of the TMA staining (note reduced numbers of tissues in Figure 21 and 22).

Cleaved caspase-8:

The staining of ccasp-8 was generally of a weak intensity and in most cases cytoplasmic with several cases demonstrating a staining of both, the cytoplasm and the nucleus, particularly in grade I and II gliomas (Figure 23). Several

observations in the evaluation of ccasp-8 staining are noteworthy: apoptotic figures were not generally positive for ccasp-8; in the majority of cases apoptotic figures were even negative for ccasp-8. When observed, mitoses often demonstrated a cytoplasmic expression of ccasp-8, while negative mitotic figures were also observed.

The levels of ccasp-8 clearly decreased from grade I and II to the high grade tumors of grade III and IV [grade I: 14% (CI 8.3-20.8%); grade II: 7% (CI 3.7-11.2%); grade III: 1% (CI 0.1-2.5%); grade IV: 2.5% (CI 1.1-4.3%); Figure 21]. The differences were highly significant from grade I to III and IV (both $p<0.0001$) and significant from grade II to III and IV ($p=0.0006$ and $p= 0.019$ respectively). The lowest levels were observed in grade III, though the differences to grade IV were not significant ($p=0.137$).

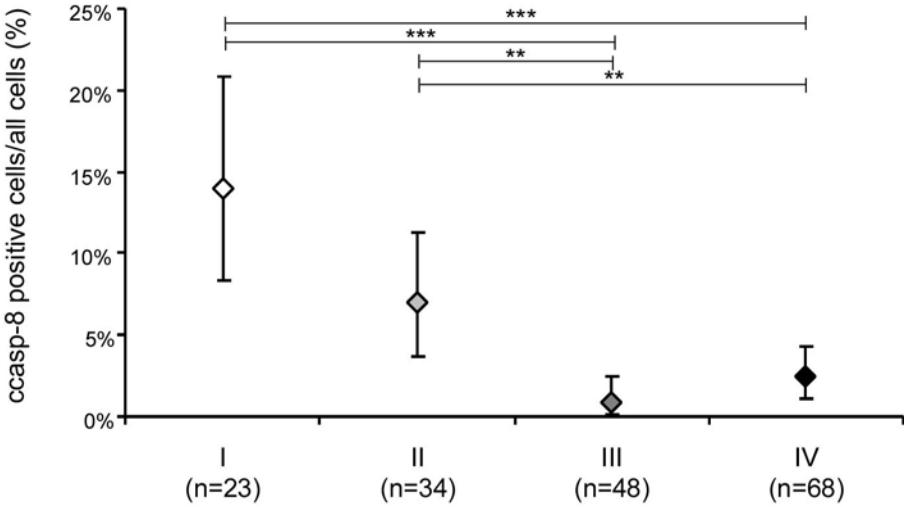

Figure 21. ccasp-8 expression in glioma grade I to IV (mean and 95% CI).

Cleaved caspase-9:

The staining of ccasp-9 was cytoplasmic and often of a weak intensity. Apoptotic figures were mostly negative, with just single exceptions to this rule and most mitoses were also negative for ccasp-9 (Figure 23).

Compared to ccasp-8, the all-over expression levels of ccasp-9 were very low [grade I: 0.3% (CI 0.2-0.6%); grade II: 0.7% (CI 0.5-0.9%); grade III: 0.8% (CI 0.6-1%); grade IV: 0.2% (CI 0.2-0.3%); Figure 22]. The expression was highest in grade II and III and the differences were statistically significant to the grades I

and IV (grade I-II p=0.018, grade I-III p=0.004, grade II-IV p<0.0001, grade III-IV p<0.0001).

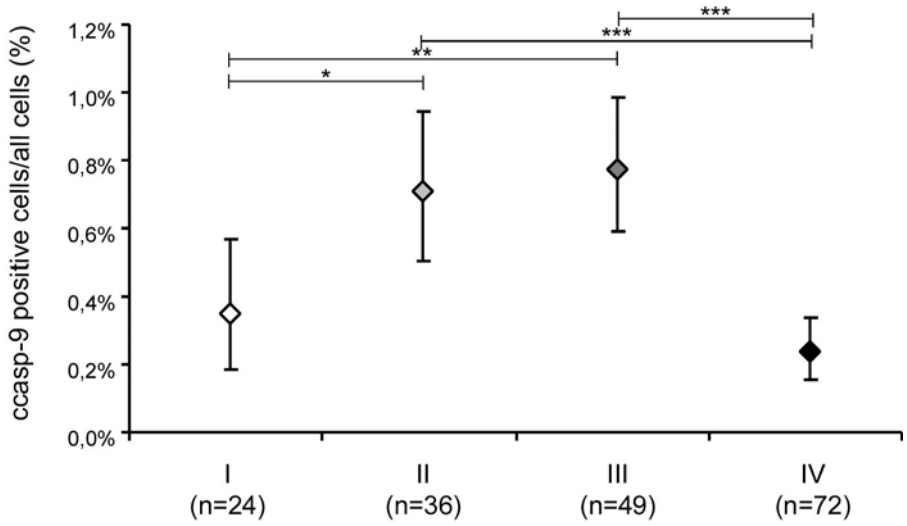

Figure 22. cCasp-9 expression in glioma grade I to IV (mean and 95% CI).

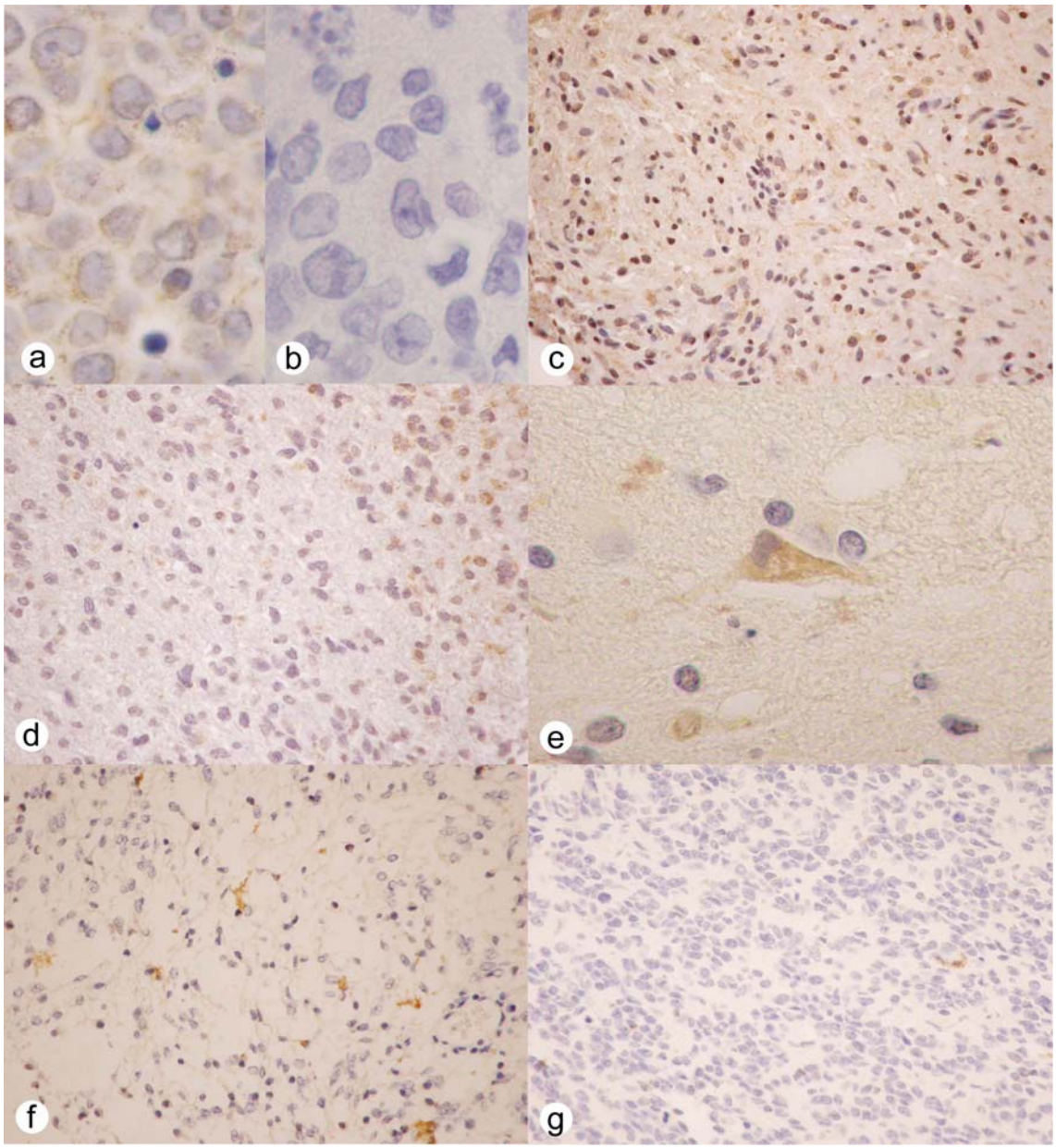

Figure 23. Micrographs of ccasp-8 and ccasp-9 immunohistochemistry in tumor tissue (immunostaining with diaminobenzidine, nuclear counterstain with haematoxylin). Positive control of ccasp-8 demonstrating a weak cytoplasmatic stain in lymphoma cells (a) and IgG_1 isotype control as negative control (b). Weakly stained tumor cells were observed in moderate levels in low grade astrocytoma, here shown for grade I (c). The expression strongly decreased in high grade tumors, here shown for grade IV (d). In general ccasp-8 negative areas dominated in high grade tumors (d on the left), while in some areas higher expression levels were observed (d on the right). Ccasp-9 staining was of an equally weak intensity. Pyknotic neurons observed in the infiltration zone

regularly demonstrated a positive cytoplasmic stain and served as an internal positive control (e). Even in low grade tumors immunostaining for ccasp-9 was rare, here shown for an above average stained grade I astrocytoma (f). In high grade tumors only single cells marked positive for ccasp-9 (g). Original magnification 1000x for a, b, e; 400x for c, d, f, g.

For both caspases, no statistically significant differences were observed concerning the infiltration zone or the relapses of glioblastoma (data not shown).

The expression of cleaved caspases-8 and -9 was also correlated with the expression of Smac, XIAP and spontaneous apoptosis detected by TUNEL for each tumor grade, but no significant correlations were obtained after adjustment of the p-values due to multiple testing with the method of Bonferroni-Holm (data not shown).

3.3 TUNEL assay

The mean cell counts per 0.25 mm² for the TUNEL assay were 781 cells in grade I, 636 cells in grade II, 1065 cells in grade III and 1524 cells in grade IV. The analysis showed very low apoptotic ratios in grade I and II tumors. The mean apoptotic counts in grade I were 0.01% (CI 0-0.042%; range from 0 to 0.4%) and 0.04% (CI 0.01-0.08%; range from 0 to 1.3%) in grade II. Both anaplastic astrocytomas and glioblastomas exhibited highly significantly more apoptotic cells (grade I vs. III $p<0.0001$; grade I vs. IV $p<0.0001$; grade II vs. III $p=0.0004$; grade II vs. IV $p=0.0009$) (Figure 24a, b and Figure 25). The apoptotic ratios were slightly higher in grade III than in grade IV with 0.17% (CI 0.1-0.19%; range from 0 to 0.6%) positive cells compared to 0.14% (CI 0.1-0.19; range from 0 to 1.6%) in glioblastoma.

The analysis of correlation revealed that neither XIAP nor Smac are correlated with spontaneous apoptosis detected with TUNEL in any of the grades I to IV (XIAP by TUNEL: grade I Spearman's rho correlation coefficient (cc) -0.1, $p=0.64$; grade II cc 0.09, $p=0.61$; grade III cc -0.2, $p=0.19$; grade IV cc 0.04,

p=0.71; Smac by TUNEL: grade I cc 0.21, p=0.33; grade II cc 0.18, p=0.3; grade III cc -0.1, p=0.51; grade IV cc -0.03, p= 0.78; data not shown).

In several high grade tumors strongly XIAP positive cells with apoptotic appearance were occasionally observed (Figure 24c, d). These apoptotic cells were often found in tumors with a weak over-all XIAP expression. In one tumor an association of these XIAP positive apoptotic figures with a pseudopalisading necrosis was observed strongly resembling the pattern of TUNEL positive foci bordering necrosis.

A correlation of TUNEL staining with the values of cellular density did not yield any significant results (data not shown).

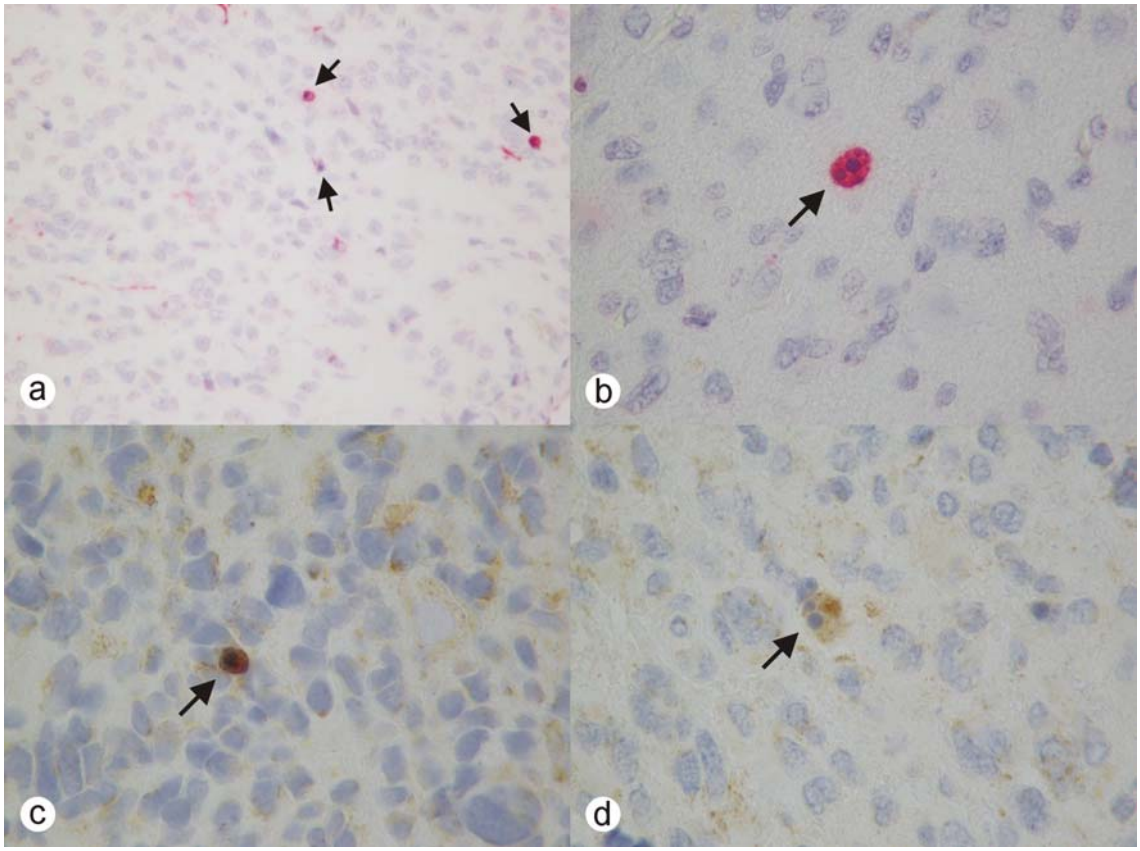

Figure 24. Representative micrographs of TUNEL staining and morphological aspects of XIAP expressing apoptotic cells (TUNEL stain with fast red, nuclear counterstain with haematoxylin; XIAP immunostaining with diaminobenzidine, nuclear counterstain with haematoxylin). (a) Positively TUNEL stained apoptotic nuclei in glioblastoma. (b) Positively TUNEL stained cell presenting typical apoptotic aspect with condensed chromatin. (c, d) Two examples of strongly

XIAP positive cells with apoptotic appearance observed in glioblastoma. These figures were occasionally observed in several high grade tumors otherwise weakly expressing XIAP. Original magnification 400x for (a), 1000x for (b-d).

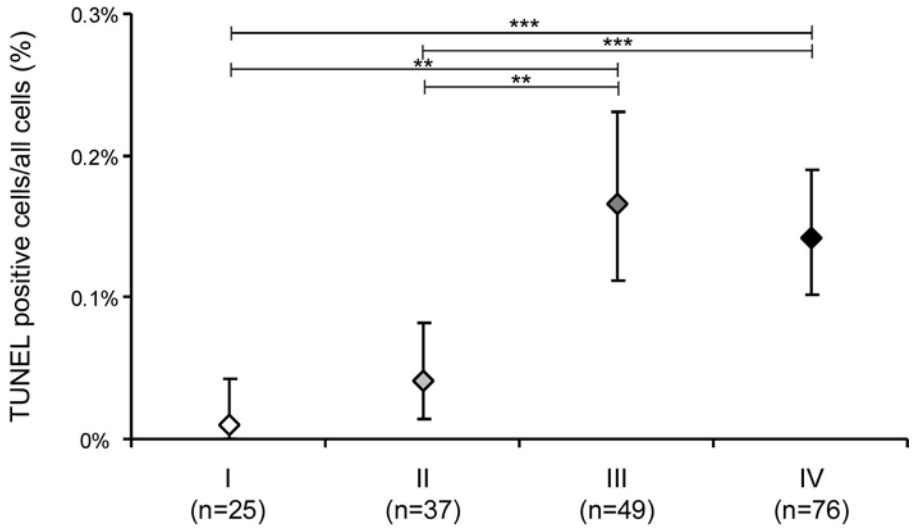

Figure 25. TUNEL positive cells in the tumors measured as percentage of morphologically confirmed labeled cells of all cells (mean and 95% CI). 0.25 mm² of each tumor were assessed. The assay shows an increase of apoptotic cells from low grade to high grade tumors.

3.4 Survival analysis

3.4.1 Survival analysis - general factors

WHO grade

One of the main reasons for the introduction and the widespread implementation of the WHO classification for tumors is the strong association of WHO grade and patient survival, thereby offering prognostic and possibly therapeutic implications. This relationship could also be demonstrated in this sample of 180 astrocytomas with survival data (Figure 26). The median survival times were 6.7 years (CI 4.2-11.4) for grade II, 3.2 years (CI 2.3-4.7) for grade III and 1.2 years (CI 0.9-1.6) for grade IV glioblastoma. As only one patient died in the group of grade I pilocytic astrocytoma, a representative survival analysis

was not possible and this grade was omitted from additional analysis. To minimize the influence of the above, further survival analysis for other factors was performed dependent on WHO grade.

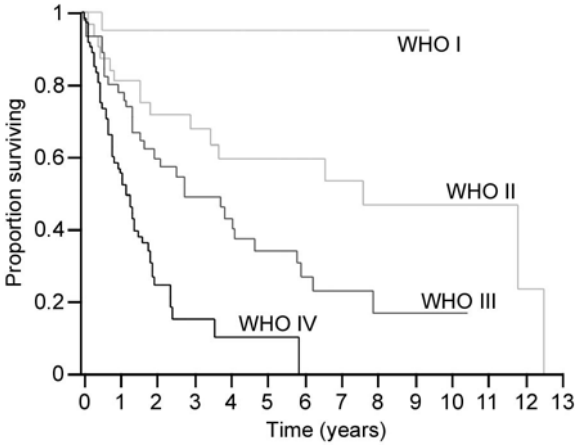

Figure 26. Kaplan-Meier analysis in relation to WHO grade.

Further general factors

Several other general factors (cellular density, relapse operations, patient sex and age) were also evaluated for a grade dependent association with survival.

While sex and cellular density showed no such properties (data not shown), a strong association of tumor reoperation and survival was detected in glioblastoma. Patients receiving at least one relapse operation had a significantly better survival than patients with no additional surgical intervention ($p<0.0001$; Figure 27). As previously described, age had a very strong association with survival in the tumor grades II, III and IV (data not shown).

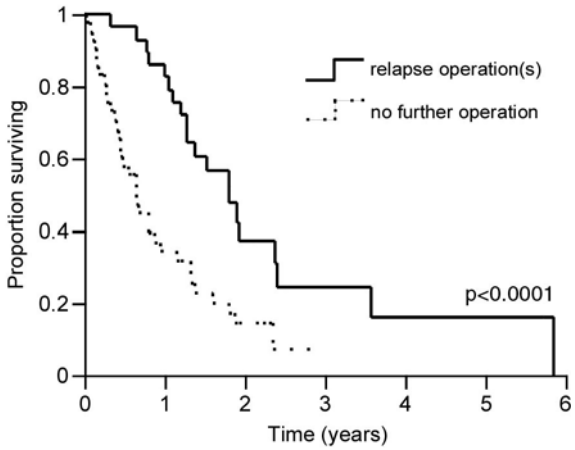

Figure 27. Kaplan Meier survival analysis of glioblastoma patients with and without relapse operation. A reoperation was associated with an improved survival.

3.4.2 Survival analysis - XIAP and Smac

No significant association of XIAP expression and survival was detected in any of the tumors grade II to IV (Wilcoxon global significance grade II: p=0.89; grade III: p=0.53; grade IV: p=0.24; Figure 28a-c). The closest approach to significance was observed comparing high and low XIAP expression in grade IV where high expression of XIAP was associated with a worse prognosis (p=0.076).

For Smac in grade II, high levels were clearly associated with a poor prognosis for the patient (high vs. mid p=0.012, high vs. low p=0.018) (Figure 28d). The median survival for the group with high expression was as low as 2.57 years (CI 1.43-5.35 years) while it was 10.36 years (CI 4.46-33.37 years) and 14.08 years (CI 5.43-56.61 years) for the group of low and mid expression, respectively. After 5 years 73% of the patients with a high expression had died, while in the group of mid and low expression only 20% were dead at 5 years post operation. In grade III, where Smac was found at levels far below the expression of other diffuse gliomas, low Smac expression was associated with the worst prognosis with a median survival of 1.48 years (CI 0.88-2.77 years) while mid expression had a median survival of 6.09 years (CI 3.15-14.17 years) and high expression 3.45 years (CI 1.97-6.89 years) (Figure 28e). In Kaplan-Meier analysis only mid

expression was significantly different to low expression, while high and low yielded no significant difference (p=0.008 and p=0.12, respectively). Finally in glioblastoma, Smac had no association with survival (Wilcoxon global significance: p=0.88; Figure 28f).

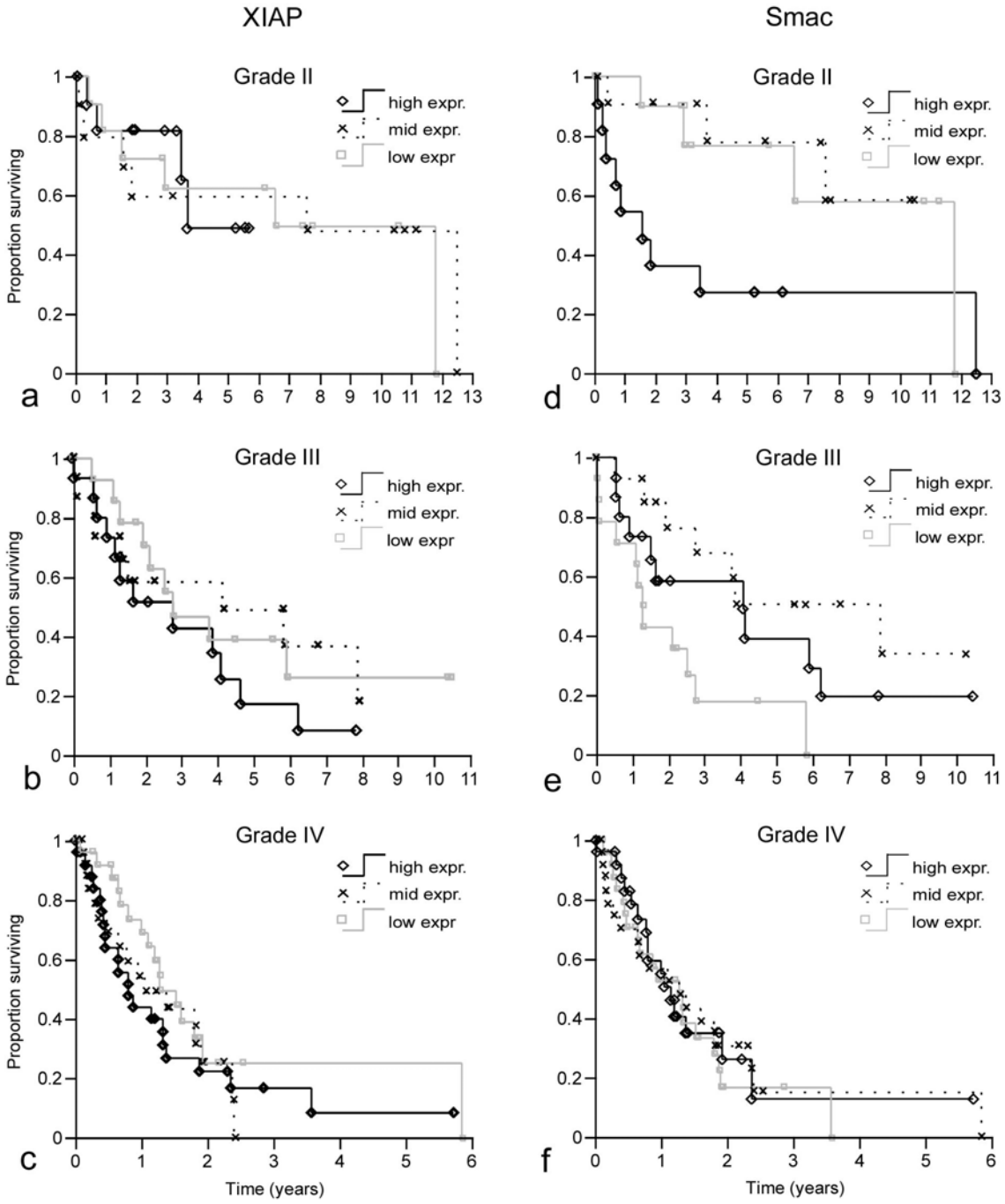

Figure 28. Kaplan-Meier analysis in relation to XIAP and Smac expression. Depicted curves (low, mid and high) represent tertiles of antigen expression of

each tumor grade. (a,b,c) No significant association of XIAP and survival could be detected in any of the grades. (d) High Smac expression displayed an association with an impaired prognosis in grade II with significant differences to mid and low expression ($p=0.012$ and $p=0.018$ respectively). (e) Low Smac had the worst prognosis in grade III with a significant difference to mid expression ($p=0.008$) but not to high expression ($p=0.12$). (f) No association of Smac and survival was detected in glioblastoma.

4 DISCUSSION

Current concepts of tumorigenesis include the hypothesis that defects in pathways regulating susceptibility to apoptosis are both involved in the development and malignant progression of gliomas and are also responsible for their intrinsic resistance to genotoxic therapy. As current approaches of breaking tumor resistance include the depletion of XIAP with its antagonist Smac or a mimic of its function the *in vivo* expression of these two proteins in astrocytic tumors of increasing grade of malignancy was analyzed and correlated to the levels of cleaved caspase-8 and -9 expression, as well as spontaneous apoptosis *(Holcik et al., 2001; Schimmer et al., 2004)*. In the following paragraphs, the above results will be discussed in the order they were primarily presented.

4.1 General findings

Among other factors, a high cellularity has been identified as a significant indicator of anaplasia in astrocytic tumors *(Kleihues and Cavenee, 2000)*. In concordance with the augmentation of anaplasia, a rise from 'moderate cellularity' in WHO II to 'increased cellularity' in WHO III and finally to 'high cellularity' in glioblastoma has been described, though for glioblastoma histopathology is extremely variable and some glioblastomas also exhibit lower degrees of cellularity. However, the mean cellular density values established in this study do not represent average values for the whole tumor tissue, as preferentially regions with a high cellularity were chosen for the construction of the TMA. This was done in accordance with the WHO postulation that grading should be performed based on areas showing the highest degree of anaplasia *(Kleihues and Cavenee, 2000)*.

A comparison of the ascertained absolute values with the literature is obstructed in several ways. Most publications actually counting tumor cells in the here presented fashion are older than the first WHO Classification of 1993 and are thus not directly comparable. More recent studies are either assessed with computer assistance as nuclear area percentage or are merely performed on

small numbers of astrocytic tumors. One such study on glioblastoma demonstrated levels of 10200 c/mm² (+/- 700 standard deviation) in 6 tumors, thus levels twice of what was detected in this study *(Brat et al., 2004).* The evaluation by Brat was performed on 6µm tissue sections, whereas the TMA slides in this study were cut at 3µm. This might in part explain the diverse results and also emphasizes the importance of tissue thickness when comparing absolute numbers in histology.

In summary, the results presented here probably represent the largest study of cellular density of astrocytic tumors classified with the new WHO Classification (2000) yielding absolute tumor cell counts.

In the WHO Classification the cellularity of pilocytic astrocytoma is described as low, compared to moderate for diffuse astrocytoma grade II *(Kleihues and Cavenee, 2000)*, whereas the here presented evaluation could not detect any significant differences in the cellularity of these entities; mean values for pilocytic astrocytoma were even above those of grade II tumors (2400 c/mm² and 2000 c/mm² respectively). It remains to be determined, whether the absolute cell count of pilocytic astrocytoma has so far been underestimated.

The area that was defined as infiltration zone revealed a mean reduction of cellularity by about 50% compared to tumor tissue, but cell count per mm² was still considerably higher than in the normal brain. This finding is indicative of glioblastoma infiltrating into the surrounding tissue. Unfortunately though, it is not possible to distinguish clearly between infiltrating tumor cells and reactive astrocytes in the adjacent tissue, as no explicit histological markers exist and both cell types may resemble each-other in appearance. Therefore, all cells of the astrocytic lineage with an pleomorph appearance were counted in this region and an interfering of reactive astrocytes with the results cannot be excluded.

Interestingly, the cellular density in recurrent primary glioblastoma was reduced, presumably as a result of cellular changes after irradiation and chemotherapy. In light microscopy cell swelling, formation of giant cells and parenchymal dispersion are common sights in relapse glioblastomas *(Ogashiwa et al., 1989)*.

These changes were also evident in several of the relapse glioblastomas of this study and strengthen the observations by Ogashiwa.

The levels of MIB-1 staining correspond well to the findings of previous studies *(Kleihues and Cavenee, 2000)*, thus demonstrating that even with the relatively small sampling size of the TMA method and the well known heterogeneity of high grade gliomas, consistent results are obtained. In line with previous results by other groups, the assessment of proliferation activity highly correlated with the cellular density in the whole group of astrocytic tumors *(Kiss et al., 1997)*. For glioblastoma the significance remained in a grade dependent analysis, indicating that cell proliferation has a major responsibility for the high cellular density exceeding the importance of the apoptotic ratio (measured as TUNEL positive apoptotic cells / all tumor cells) as this did not show any correlation to the cellular density (also see below). Thus, it is likely that the improved mitotic activation is of more effect concerning the cellular density than the impairment of apoptosis in high grade tumors. For grade III astrocytoma the situation seems to be similar, while the cellularity of lower grade tumors did not show any correlation to either proliferation or apoptotic ratio.

4.2 XIAP expression in tumor tissue and normal brain

The evaluation of XIAP revealed that an increase of protein expression level was closely associated with an increase of malignancy. XIAP, which is known to interact with several pathways associated with malignant progression, may contribute to the formation of the malignant phenotype which varies greatly from low to high grade. The increase of XIAP in accordance to the WHO grade might be useful as an additional diagnostic tool at first tumor occurrence, akin to other immunohistochemical markers like MIB-1 (Ki-67) which also show a close correlation to the grade of malignancy *(Raghavan et al., 1990)*. 90% of high grade gliomas show an immunoreaction for XIAP, further strengthening the importance of an anti-XIAP therapy. As in low grade glioma overall expression is sparse and up to 36% exhibit no XIAP, these modalities seem less favorable, although individual tumors of grade II have a high expression of up to 77%

positive cells, indicating that a XIAP inhibiting therapy option might also exist for a subgroup of patients with low grade tumors.

Interestingly, XIAP was not detectable in neuronal cells, not even when in close proximity to the tumor in the infiltration zone. This indicates that neurons surrounding tumors might utilize XIAP independent ways of apoptosis regulation, possibly by other IAP members like NAIP, the neuronal apoptosis-inhibitory protein *(Robinson, 1995)*. A XIAP specific therapy might therefore have the additional advantage of not interfering with the apoptosis regulation of neurons. In contrast, Vischioni et al. recently described XIAP protein expression exclusively in large cortical neurons of the layers 3 and 5 while other neurons did not express XIAP in normal human brain, indicating that the above conclusion has to be viewed with caution *(Vischioni et al., 2006)*.

This study of course remains a sole observation of the in vivo protein expression and it is above the scope of the data to give answers of the effect of an anti-XIAP therapy in humans. The results obtained from cell culture and animal experiments however give reason to believe in a positive effect of depleting XIAP. Under the assumption that the results are transferable to human tissue this would mark an improvement to the available therapies of gliomas.

4.3 Smac expression in tumor tissue and normal brain

As over all low apoptotic ratios have been described in glioblastomas and no relationship of Smac with spontaneous apoptosis or expression with caspase-8 or -9 was detectable, the high levels of Smac in grade IV neoplasms suggest that the protein fails to induce apoptosis in these tumors. In glioblastoma mechanisms blocking the release of pro-apoptotic factors from mitochondria have been described, thereby enhancing the cells' resistance to apoptosis *(Cory et al., 2003)*. It is possible that Smac is also blocked from its release into the cytoplasm and accumulates in the mitochondria thus resulting in the high levels in glioblastoma without an induction of apoptosis. Interestingly, Smac levels were reduced in grade III glioma compared to other diffuse gliomas, indicating that differences of apoptosis escape might exist between anaplastic

astrocytoma and primary glioblastoma. In line with previous findings, the presented results suggest the preference of a cytosolic form of Smac protein to induce apoptosis in a therapeutic approach, as endogenous Smac might well be locked into mitochondria in the majority of glioblastoma *(Fulda et al., 2002b)*.
The high expression of Smac protein in neuronal cells especially in the close proximity of the tumors may well reflect a reaction of these cells to apoptosis inducing stress. Neuronal cells are known to be highly sensitive and to react fast to various forms of cellular stress like hypoxia, oxidative stress and toxic chemicals. Experiments in rodents observed only a negligible amount of Smac in both the mitochondria and the cytosol of neurons in the normoxic state but an increased Smac expression in the cytosol 5 hours after an hypoxic event *(Shibata et al., 2002)*. It remains unclear whether the high Smac expression in the neurons of this study is due to preterminal hypoxia (normal brain) and hypoxia during extraction (tumor tissue) or some other form of cellular stress.

4.4 XIAP and Smac expression pattern in the infiltration zone

It was shown that XIAP is highly expressed in the infiltration zone of glioblastoma. The infiltration zone was defined as an area adjacent to the tumor with a clearly reduced cellular density and no further signs of atypia like vessel abnormalities or necrosis. In diffuse astrocytoma this area is pervaded by migratory tumor cells *(Pilkington, 1994)* and there is evidence that signals inducing cell invasion also promote cell survival by suppressing apoptosis of migratory cells *(Cho and Klemke, 2000).* This process is likely to be associated with an increase of XIAP levels, explaining the high XIAP levels in the infiltration zone. Smac was expressed at a significantly reduced rate in the infiltration zone. This can also be seen as a reduction of responsiveness to pro-apoptotic stimuli in migratory tumor cells. Additionally, a strong correlation of individual XIAP levels in tumor and infiltration zone and in normal brain grey and white matter was demonstrated, indicating a supra-regional regulation of this protein. The results for XIAP expression strongly resemble the expression pattern of MIB-1. The demonstration of a high regional correlation of the proliferation marker is in line with the work of others *(Onda et al., 1994)* and is indicative that

despite histopathologically evident areas of improved proliferation the all-over levels of proliferation are relatively steady and thus may constitute an immanent characteristic of a specific glioblastoma. By contrast, an oral communication on the regional expression pattern of VEGF *(Goeppert, publication in preparation)* revealed no correlation for this factor (p=0.24) which is consequential to the previously described induction by hypoxia with high expression in the immediate proximity to necrotic foci *(Shweiki et al., 1992)*. It is indicative that XIAP expression is not subject to regional protein induction such as VEGF *(Shweiki et al., 1992)* but is rather induced in a supra-regional manner akin to MIB-1 *(Onda et al., 1994)*. As XIAP also demonstrated a strong correlation in white and grey matter of normal brain, this mechanism seems to function in tumor tissue and normal brain. Smac on the other hand showed correlation neither in tumor nor in normal brain, suggesting a different form of regulation, possibly through factors in the direct environment.

The question remains whether this observation is of any relevance. The reason the issue was investigated is, that molecular analyses of microdissected glioblastomas demonstrated a genetic polymorphism with genetically diverse areas within the tumor of single patients *(Cheng et al., 1999)*. This is not a contradiction to the monoclonal origin of tumorigenesis, but indicates that genetic instability may produce tumor cell offspring with varying genetic contents. For evaluating the theoretical feasibility of an anti-XIAP therapy it was considered important that the protein is not merely expressed in regionally confined areas but rather in a widespread fashion. This indicates that the genetic alteration leading to its increase is a primal event of tumorigenesis. The high degree of correlation of XIAP in the tumor center and the periphery suggests that this protein represents an immanent characteristic of a particular glioblastoma akin to MIB-1 (Ki-67).

4.5 XIAP and Smac in relapse glioblastoma

The expression of Smac and XIAP was analyzed in 25 relapse glioblastomas and their corresponding primary tumors. Though no significant differences could be detected, Smac was expressed at lower levels in relapse and the difference

was nearly significant (p=0.054). This observation suggests that apoptosis regulation might change from primary to relapse glioma, possibly contributing to the commonly even higher degree of resistance towards genotoxic therapy observed in relapse tumors *(Brandes, 2003)*. As the majority of the patients received radiation and chemo-therapy after their first operation, it is possible that the observation reflects an effect of cell selection. Smac overexpressing glioma cells have been shown to be more susceptible to various genotoxic stimuli *(Fulda et al., 2002a; Roa et al., 2003)*, and this might be the same with *in vivo* tumor cells expressing high levels of endogenous Smac. This effect could lead to the depletion of the susceptible tumor cells, resulting in a tumor more resistant to genotoxic therapy. Interestingly, no changes of XIAP expression were detected, indicating that this protein is not subject to interference by genotoxic therapies.

4.6. Correlation of Smac and XIAP expression with age, sex, cellular density and proliferation

A significant increase of XIAP protein with age could be detected exclusively in glioblastoma. This is especially interesting, as old age has been identified as a major predictive factor for the prognosis of glioblastoma. Various other changes in protein profiles in these tumors have so far been observed in conjunction with age, foremost a reduction of p53 mutation and an increase of EGFR amplification *(Batchelor et al., 2004; Ohgaki and Kleihues, 2005; Sarkar et al., 2004)*. These observations feed the discussion of age dependent genetic subtypes in glioblastoma, proposing a role of XIAP in the highly malignant course of glioblastoma in old age.

The results of this study are of additional relevance, as great care was taken not to include secondary glioblastomas in the sample. For these tumors a different genetic background with an increased expression of mutated p53 and a female gender predominance has been reported (*Ohgaki and Kleihues, 2005)*. It would be interesting to see whether XIAP expression also differs in primary and secondary glioblastomas.

A strong negative correlation of Smac and cellular density was detected in glioblastoma. As in every non-experimental correlation of two factors (A and B) at least three possible explanations for the correlation exist: Either A is the cause of B (i), B is the cause of A (ii) or there is no causal connection as a third factor underlies both A and B (iii). In this setting the above could take the following form: (i) Elevated levels of Smac lead to an enhancement of apoptosis and/or the inhibition of clonogenic growth resulting in low cellular density. (ii) Smac is up-regulated by conditions of high cellular density, not necessarily leading to the induction of apoptosis and not having an effect on cellularity. (iii) Unknown super ordinate regulators might control both expression of pro-apoptotic Smac and cellular density, while there is no direct interaction of these two parameters.

While (i) seems to be the most intuitive explanation, no further evidence could be found for this theory. Smac did not correlate with the rate of spontaneous apoptosis nor the rate of proliferation. As stated in the introduction, apoptosis is a kinetic event of rather short duration and apoptotic cells “disappear” rather rapidly. This leaves the option that elevated levels of Smac might still lead to higher rates of apoptosis but might not be detectable by the means used here.

The earlier discussed supposed regional regulation of Smac protein (see 4.4) militates in favor of the second theory. It seems possible that the harsh environment or the deprivation of cellular resources in conditions of elevated cellularity leads to an up-regulation of pro-apoptotic Smac. Possibly other factors such as deregulated members of the Bcl-2 family then disrupt the release of Smac, thereby inhibiting the actual induction of apoptosis (*Cory et al., 2003)*.

The third theory is hard to establish with the experimental set-up of this study. Still, there is some evidence that super ordinate regulators exist. Other proteins like pro-apoptotic Endonuclease-G and the cellular surface protein HLA-E have also been observed to negatively correlate with cellular density *(Mittelbronn and Herzog, manuscript in preparation)*. This at least makes the direct causal connection of Smac and cell density unlikely and implies the existence of underlying modes of regulation. These superordinate regulators might induce

proliferation (thereby leading to high cellular density) and at the same time suppress a broad spectrum of proteins (like Smac, Endonuclease-G and HLA-E). Though XIAP exhibits an experimental connection with both mitosis and the degradation of Smac, the results do not indicate that XIAP functions in such a superordinate role as no correlation of XIAP with proliferation, the expression of Smac or cellular density was observed in glioblastoma.

4.7 Correlation of XIAP and Smac expression

No correlation of XIAP and Smac protein was detectable in any of the tumor grades. This is to some extent surprising, as a mutual degradation of the two proteins has been described (*Fu et al., 2003; MacFarlane et al., 2002; Roberts et al., 2001)*. One reason for the lack of interaction might be that in this study an antibody detecting full length Smac was used, while Fu et al. have shown that only a splice variant of Smac (Smac3/Smac δ) actually accelerates the auto-ubiquitination and destruction of XIAP. It is unlikely that the antibody also detected the splice variant, as the specific reactivity has been confirmed via Western Blot analysis in several cell lines *(Zymed Laboratories, South San Francisco, USA)*. On the other hand a potent degradation of Smac has been demonstrated to be promoted by XIAP in cell cultures (*MacFarlane et al., 2002)*. The results presented here could not confirm a measurable effect of this in vitro observation in human glioma tissue.

A further point of discussion concerning the lack of antibody correlation is the uncertainty of Smac localization mentioned above (see 4.3). It seems likely that in high grade glioma the interaction of XIAP and Smac might be hampered by the proteins' localization in two distinct compartments (XIAP in the cytosol, Smac in the intermembrane space of the mitochondria). If the release of Smac is inhibited it is evident that no further interaction can take place with XIAP.

4.8 Expression of cleaved caspase-8 and -9

Cleaved caspase-8:

The levels of ccasp-8 as a marker of the activity of the death receptor pathway have not been well characterized in glioma tissue. A recent study demonstrated

very low to undetectable levels of Caspase-8 in several diffuse astrocytic tumors via Western blots (3 grade II, 5 grade III and 11 grade IV tumors) with no relation of the expression to clinical grade, patient age or sex *(Ashley et al. 2005)*, while Bodey et al. analyzed several high grade gliomas occurring in childhood for the inactive form of caspase-8 and observed more than 10% of astrocytic tumor cells with a high intensity staining (n not specified; *Bodey et al., 2004).* In concordance to the former results low levels of ccasp-8 were detected in high grade tumors, while the study presented here also includes a large number of diffuse astrocytoma grade II and pilocytic astrocytoma. Interestingly, the active form of caspase-8 was detected at moderate levels in the lower grades, demonstrating a massive down regulation of ccasp-8 from low grade to high grade astrocytic tumors.

As Bodey was able to detect considerable amounts of inactive full length caspase-8 in glioma tissue, two possible explanations for the reduction of the cleaved form seem reasonable: either, (i) the activation of caspase-8 is inhibited or (ii) the active form of caspase-8 is rapidly degraded.

(i) The activation of caspase-8 is induced through various death receptors, which are in turn activated by members of the tumor necrosis factor family such as TNFα, Fas Ligand and the recently described TNF-related apoptosis-inducing ligand (TRAIL). For the latter, an activation through death receptor 4 and 5 has been described in glioma cell lines, leading to the recruitment of Fas-associated death domain (FADD) which consequently leads to the attraction of caspase-8 and a subsequent activation of this caspase *(Hawkins, 2004)*. Two isoforms of c-FLIP (cellular FADD-like IL-1β-converting enzyme-inhibitory protein) have been identified as negative regulators for the activation of caspase-8 *(Xiao et al., 2002)* and a reduction of c-FLIP leads to TRAIL sensitivity of resistant glioma cell lines *(Song et al., 2000).* The extrapolation of these *in vitro* observations to our results suggest, that the inhibition of caspase-8 activation is a process predominantly occurring in high grade astrocytoma, whereas tumors of a lower grade may lack such inhibitory mechanisms.

(ii) Rapid binding and protein degradation of ccasp-8 could also lead to reduced levels in high grade tumors, but even though the expression of XIAP and ccasp-

8 seems to be in diametrical opposition, the reduction may not be directly attributed to XIAP. It has been demonstrated repeatedly, that while XIAP binds and blocks several members of the caspase family it does not bind caspase-8 *(Deveraux et al. 1998; Datta et al., 2000)*. Equivalent results have been obtained for other members of the IAP family *(Deveraux et al. 1998, Tamm et al., 1998).* In contrast, Wagenknecht et al. could demonstrate that adenoviral XIAP gene transfer blocked caspase-8 processing in two glioma cell lines *(Wagenknecht et al., 1999)*. In the study presented here the results indicating that XIAP does not directly interfere with ccasp-8 are strengthened, as the protein levels of the two proteins do not correlate in a grade dependent manner. In this respect, the lacking correlation of Smac and ccasp-8 is in accordance to the above observations, as Smac mainly activates the various caspases by inhibiting XIAP.

It has been argued, that the low levels of caspase-8 in glioma may complicate the future development of death ligand-based therapies *(Ashley et al. 2005)*, while our results in conjunction with the results of Bodey indicate, that the problem is not a general lack of caspase-8 but rather the disruption of caspase-8 activation. Further strategies concerning apoptosis induction via the death receptor pathway should concentrate on blocking the inhibition of caspase-8 activation, rather than solely inducing caspase-8 protein.

A main mode of apoptosis promotion by caspase-8 is by the activation of effector caspase-3 *(Thorburn, 2004)*. Several studies demonstrated exceedingly low levels of activated caspase-3 in glioma: Riemenschneider detected cleaved caspsas-3 only in single cells in glioblastoma (n=29), Uno could not detect any cleaved caspase expression in 83% of diffuse astrocytoma WHO II (n=12), no cleaved caspase-3 in all investigated anaplastic astrocytoma (n=10) and none in 79% of investigated glioblastomas (n=34), and Brat detected as low as 0.02-0.2% positive tumor cells in tissue adjacent to pseudopallisading tumor cells in 9 glioblastomas *(Brat et al., 2004; Riemenschneider et al., 2006; Uno et al., 2006).* These levels are clearly below what was observed for cleaved caspase-8 in this study, indicating that ccasp-8 does not lead to a measurable casase-3 activation in all events. This ‘gap of caspases’ is indicative of a potent inhibition

of cleaved caspase-3 most likely by members of the IAP family and especially by XIAP, of which this study could demonstrate high levels in astrocytic tumors.

Cleaved caspase-9:

Caspase-9 is activated during apoptosis and associates with Apaf-1 and mitochondrial cytochrome c, thereby forming the apoptosome. This pro-apoptotic complex has been shown to cleave and activate downstream effector caspases such as caspase-3 *(Cain et al., 1999; Li et al., 1997; Zou et al., 1999)*. The process of caspase-9 activation can be interrupted by cytoplasmic XIAP if it is not itself bound by Smac or Omi/HtrA2 which sequester free XIAP and thereby prevent it from interacting with the apoptosome complex *(Twiddy et al., 2004)*.

The all-over expression of the active form of caspase-9 in the presented study was very low, with its lowest expression of merely 0.2% positive tumor cells in glioblastoma. Despite the low expression a significant reduction of the levels from low grade diffuse astrocytoma and anaplastic astrocytoma to glioblastomas was observed.

Two possible explanations for the reduction are shortly discussed: First, ccasp-9 is a direct binding-partner of XIAP, for which highest levels were demonstrated in glioblastoma. It seems likely, that the high concentrations of XIAP inhibit ccasp-9, although a statistically significant grade dependent correlation of the two factors was not observed.

Secondly, the above discussed mechanism of blocking the release of mitochondrial factors (see 4.3) could also affect the levels of ccasp-9, as its activation depends on the interaction with mitochondrial cytochrome c which is releases after the initiation of the mitochondrial pathway of apoptosis initiation. The inhibition of cytochrome c release would therefore lead to low levels of ccasp-9.

It can be assumed, that the two processes would not exclude one another, rather they might amplify each other, thereby keeping the levels of glioblastoma ccasp-9 at such low levels.

Taken together, it is evident that in glioblastoma both caspases that are directly inhibited by XIAP, namely cleaved caspase-3 and cleaved caspase-9 as well as spontaneous apoptosis detected by TUNEL demonstrate similar expression levels of around 0.2% [ccasp-9: 0.2% (CI 0.2-0.3%); ccasp-3: 0.02-0.2% (Brat et al.); TUNEL: 0.14% (CI 0.1-0.19%)] while the levels of XIAP independent ccasp-8 are more than tenfold higher [2.5% (CI 1.1-4.3%)]. Thus, despite the lack of direct correlations, a dependence of the apoptosis regulating factors ccasp-9, ccasp-3, XIAP and the rate of apoptosis (TUNEL) seems likely.

4.9 TUNEL assay

In line with previous observations low over-all levels of spontaneous apoptosis were detected in gliomas with an increase in high grade glioma, though the results are clearly towards the lower end of what other authors have described *(Heesters et al., 1999; Kordek et al., 1996; Ralte et al., 2001)*. This might be in part due to the fact that in the presented study TUNEL positive cells were only counted as apoptotic when clearly confirmed by morphological criteria of apoptosis. This form of counting is in line with the instruction manual of the TUNEL kit and has also been proposed by Darzynkiewicz (*Darzynkiewicz et al., 1992).* The levels of cleaved caspase-3, an equally accepted marker for apoptosis in paraffin embedded tissue *(Gown et al., 2002),* are very close to what was detected with the TUNEL assay in this study, thereby further strengthening the results [TUNEL in glioblastoma 0.14 % (CI 0.1-0.19); cleaved caspase-3 0.02-0.2% *(Brat et al, 2004)*].

In this study the level of spontaneous apoptosis did not correlate with the protein expression of pro-apoptotic Smac nor of anti-apoptotic XIAP. Several other authors have described a similar lack of a relationship with the immunoreactivity of important regulators of apoptosis like p53, PTEN, Bcl-2 and Bax *(Ellison et al., 1995; Kordek et al., 1996; Korshunov et al., 1999; Nakamizo et al., 2002; Tews, 1999)*. Thus, the results confirm previous conclusions that the network of apoptosis regulating factors may be too complex for clear associations with single factors. Other authors have proposed that the increased levels of apoptosis in high grade gliomas might be a consequence of

elevated proliferative activity resulting in a more prominent tumor hypoxia *(Steinbach and Weller, 2004)*. Interestingly these possible pro-apoptotic stimuli did not lead to a cleavage of the initiator caspases-8 and -9 and despite a reduced activity of both, the mitochondrial and the death receptor pathway, the rate of spontaneous apoptosis increases with the grade of malignancy. This raises the questions of further pathways of apoptosis initiation and execution. Possible candidate factors that might be important in caspase-independent apoptosis are AIF and Endonuclease-G *(Kim et al., 2005*). In a recent study of our group on astrocytic tumors an increase of both, AIF and Endonuclease-G in high grade tumors was observed, possibly compensating for the lack of cleaved caspases *(Mittelbronn and Herzog, manuscript in preparation)*. A further question beyond the scope of this study is, if besides apoptosis, alternative forms of regulated cell death exist in astrocytic tumors. There is increasing evidence, that other types of cell death like necroptosis and autophagy may constitute additional forms of cell death with possible importance in cancer *(Degterev et al., 2005)*.

4.10 Survival analysis

Several items should be addressed briefly before the actual discussion of the association of survival with XIAP and Smac.

As patient death was chosen as the end point, pilocytic astrocytoma could not be included into the survival analysis. It is clear that for a disease with a 10 years survival of 96% either very large numbers of patients have to be integrated or a different end point such as relapse of disease has to be chosen. No data concerning disease relapse of low grade or progression from grade II to high grade was acquired, so no other end point was realizable.

Patient categorization followed the criteria stated in 2.1 and no further patient selection was performed. Thus, the sample forms a representative group of astrocytic tumor patients who underwent operation within a period from 1993 to 2003, although the frequency of occurrence, especially of lower grade tumors is not proportionate. It is important to note that this sample only represents patients undergoing operation. As stated in the introduction, epidemiological

studies demonstrated that only 54% of glioblastoma patients undergo surgical intervention *(Ohgaki and Kleihues, 2005)*. The same investigations also described a highly reduced median survival of just 0.4 years for glioblastoma patients compared to 1.2 years observed in this study. For a truly representative sample, around 30% of the glioblastoma patients should be over 70 years while this was only the case for 16% of the sample. The presented survival of grade III was also considerably higher than in the above epidemiological investigation (3.2 years vs. 1.6 years respectively) while the results for grade II were fairly close (6.7 years vs. 5.6 years, respectively).

Besides the well established influence of patient age in astrocytoma *(Chang et al., 2001; Kallio et al., 1991)* the performance of relapse operations was also identified as a positive survival factor. The observed beneficial effect is most likely to be strongly influenced by the tumor localisation and the preoperative Karnofsky Performance Scale. This scale allows patients to be classified as to their functional impairment and is crucial in the decision of further surgical intervention or other therapeutic options. It has been demonstrated to have independent prognostic properties in glioblastoma *(Curran et al., 1993)*. An interesting question for further investigations could be to evaluate the exact origin of the survival benefit of the Karnofsky index. As indicated by the above observations, the benefit might be strongly dependent on the performance of further interventional modalities.

Cytoplasmic expression of XIAP did not exhibit a concrete association with survival. This should however not lead to the reverse conclusion, that patients would definitely not benefit from artificial lowering of XIAP. Several studies have already demonstrated beneficial effects on survival in animal models of various tumors *(Fulda et al., 2002a; Hu et al., 2003)* and now that a widespread expression of XIAP protein in glioma has been demonstrated, further investigation should concentrate on feasible methods of lowering this protein in human tumors.

The survival analysis of Smac revealed some unexpected results. While Smac was clearly not associated with survival in glioblastoma, grade II astrocytoma patients with high Smac expression had the worst prognosis and this remained

significant in conjunction with age. Five gemistocytic astrocytomas, which have been described to have a worse prognosis, were included in the sample of this study *(Kleihues and Cavenee, 2000)*. These did not show a different Smac expression and Smac remained a negative predictor of survival when they were omitted from analysis. The reason for this negative association remains unclear. The most frequent cause of death for grade II astrocytoma is the progression to a higher grade tumor, most commonly to grade IV *(Kleihues and Cavenee, 2000; Ralte et al., 2001)*. The high levels of Smac in grade IV and the reduced survival of grade II with high levels of Smac make it possible that elevated expression of Smac in low grade tumors heralds the progression to a secondary glioblastoma, thus drastically reducing patient survival. Further studies evaluating the actual degree of tumor progression and the expression of Smac in secondary glioblastoma are needed to verify whether a high expression in low grade tumors is an indicator of malignant transformation.

In both grade III and IV Smac protein exhibited no significant association with survival. Similar results were observed in a group of high grade gliomas for the pro-apoptotic mitochondrial protein ARTS which has also been described to interact with XIAP *(Gottfried et al., 2004)*. Even though underlying associations might be blurred as important influence factors such as tumor localisation and patient treatment were not taken into account, a direct association of the proteins and survival seems unlikely. As previously discussed for apoptosis, it is likely that the circumstances leading to patient death might be too complex for clear associations with the expression of single factors.

4.11 Conclusion and outlook

In conclusion, it was shown that XIAP is widely expressed in high grade glioma and increases with the grade of malignancy of astrocytic tumors. Strong correlations of XIAP expression were observed in different regions of individual patients indicating that the protein is likely to be an immanent characteristic of a particular glioblastoma. XIAP also demonstrates an increase with patient age in glioblastoma and cells in the infiltration zone were strongly expressing XIAP. Despite its lack of correlation with apoptosis and association with patient survival the above findings clearly mark XIAP as a potential target for anti-tumor therapies. It is important to keep in mind that none of the patients in this setting underwent an anti-XIAP therapy and that thus a lack of association with survival does not indicate an ineffectiveness of such modalities.

Smac is also highly expressed in astrocytic tumors and its expression indicates a different mode of apoptosis escape in grade III and grade IV tumors. Smac expression seems to depend on regional regulation and cellular density or a super ordinate factor regulating both Smac expression and cellular density. As Smac does not correlate with spontaneous apoptosis the pro-apoptotic protein seems to fail its function in astrocytic tumors. Still, there are indications that Smac is pertinent in the susceptibility to genotoxic therapies as indicated by its reduction in relapse tumors.

It was shown, that the expression of cleaved caspase-8 and -9 are both reduced in high grade gliomas, indicating that both pathways of apoptosis initiation are inhibited, while both Smac and XIAP did not demonstrate a measurable association with the levels of caspases. In contrast, the levels of TUNEL positive apoptosis increased in high grade tumors, indicating, that further pathways leading to apoptosis exist and play an important role in high grade glioma.

It was further demonstrated that the association of the enhanced proliferation rate on cellular density is superior to the association with the rate of apoptosis, raising general questions about the importance of apoptosis resistance in astrocytic tumors and about its proclaimed therapeutic potential.

As an outlook, several suggestions for future studies are summarized below:

- The expression of Smac offers several threads for future studies:
    - The results presented here were obtained with one method of protein detection (immunohistochemistry) and before further studies are conducted a reproduction with a further method is required (e.g. Western blot). Especially the marked difference of grade III and IV should be reproduced by at least one different method.
    - For the evaluation of the function of Smac the cellular localization in astrocytoma is of great importance. Immunogold methods with electron microscopy as well as Western blotting of the separated cytoplasmic and mitochondrial compartment could be used to identify the exact localization in vivo.
    - Primary cell cultures of high grade gliomas could help to verify the suspected block of Smac release by measuring the protein contents in the cytoplasmic fraction before and after apoptosis initiation. A quantification of Smac protein after subtoxic chemical stimuli or oxygen deprivation could clarify questions concerning the regulation of Smac. Possible questions include whether Smac is induced by such stimuli or if, as indicated by the signified reduction in relapse tumors, a selection of cells with low Smac levels can be observed.
    - The association of survival and Smac in grade II, indicating possible properties as a marker of malignant progression, should be further evaluated. An extended tumor bank with an emphasis on low grade tumors with progressed relapses reproducing the results would be a first step, but ultimately a prospective study should be aspired.
    - As Smac is just one among several apoptosis inducing factors, further studies are needed to validate the general importance of Smac in relation to other regulators of apoptosis like cytochrome c, HtrA2/Omi, Endonuclease G, AIF, ARTS and to see whether a wider panel of apoptosis inducing factors demonstrates a verifiable correlation to in vivo apoptosis. This could also clarify if there are general differences

in the regulation of apoptosis also including other regulators of apoptosis in grade III and grade IV tumors.

- The results of XIAP also suggest several further investigations:
    - As stated for Smac, a reproduction of the results with a different method is needed to validate the in vivo expression of XIAP.
    - As anti-XIAP therapies seem promising, further studies should then concentrate on methodological aspects for the application of such modalities. The possible side effects of XIAP lowering agents especially on neurons should also be evaluated in an *in vivo* model.
    - As the results indicate a suppression of pro-apoptotic factors in infiltrating cells, migration assays could be used to evaluate a possible differing expression of Smac and XIAP in this population. This is of interest as new therapeutical approaches should especially address infiltrating cells. Elevated levels of XIAP in this population would make anti-XIAP therapies all the more attractive.
    - XIAP did not correlate to apoptotic ratios (TUNEL) or to the expression of ccasp-9. As these correlations are established in cell cultural experiments, it is most likely that such kinetic events as the interaction of two proteins or the initiation of apoptosis, evade immunohistochemical detection. The observation that both the mitochondrial and death receptor pathway are inhibited in high grade tumors while apoptotic ratios increase, hints at further pathways leading to apoptosis. As indicated above, functional investigations in this area should be performed in cell cultures.
    - The age related expression of XIAP in glioblastoma also demands further investigation. Is it possible to identify a common genetic origin for the various changes of old age glioblastoma, especially the amplification of EGFR and increase of XIAP, thereby confirming evidence of an age dependent genetic subgroup of glioblastoma? What other reasons for the differential expression can be imagined? Is the increase of XIAP secondary to the amplification of EGFR,

possibly via nuclear factor-κB? Could a differential immune surveillance or hormone balance of old age lead to a different expression of tumor proteins? The evaluation of these questions requires the age dependent analysis of further tumor associated proteins and should also include genetic analysis.

5 ABSTRACT

Recent studies demonstrate that defects in apoptotic pathways are involved in both the development of astrocytomas and in their intrinsic resistance to genotoxic therapies. A major role in apoptosis regulation has been demonstrated for XIAP. Animal and *in vitro* glioma studies encourage therapeutic attempts at depleting XIAP with its endogenous antagonist Smac or mimics of its function. In this study the protein expressions of XIAP and Smac in tissue micro arrays of 194 astrocytomas of different WHO grade (I-IV) were immunohistochemically analyzed. XIAP expression was found to increase significantly with the grade of malignancy, while Smac was expressed at the lowest levels in grade III tumors, whereas the highest expression was observed in glioblastoma. XIAP protein was generally not observed in neuronal cells, while Smac was strongly expressed in neurons of the normal brain and the infiltration zone of glioblastoma. Further, a significant correlation of patient age and XIAP expression was identified exclusively in glioblastoma. The two proteins exhibited a differing distribution in the tumor tissue. While the expression of XIAP was highly correlated in the tumor center and the periphery, Smac showed no correlation. This was interpreted as an evidence for the differing regulation of the two proteins, XIAP expected to be an immanent characteristic of the tumor while Smac seems to react to regional stimuli. A correlation of both proteins with cleaved caspase-8 and -9 as well as the rate of apoptosis as detected by the TUNEL assay could not assess any direct associations. Anti-apoptotic Smac revealed a significant association with survival in WHO grade II with high levels linked to a worse prognosis. XIAP displayed no association with survival in WHO grade-dependent Kaplan-Meier analysis. In conclusion, it is shown that XIAP is significantly upregulated in high grade glioma, confirming the feasibility of an anti-XIAP therapy. The Smac expression profile indicates that differences of apoptosis escape might exist between anaplastic astrocytoma and primary glioblastoma.

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7 APPENDIX

7.1 Acknowledgments

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