Abstract. During the physiological process of PCD, the cell initiates a sequence of events culminating in the disintegration of the cell into small, membrane-bound apoptotic bodies. The intrinsic part of the PCD program arises from the mitochondria when it releases cytochrome c from the mitochondrial intermembrane space into the cytosol, forming the caspase-activating complex or apoptosome. The family of caspases is involved in the execution of genetically controlled PCD. Caspase-3 is expressed in normal and neoplastically transformed human cells and, like other caspases, is synthesized as an inactive, 32kDa proenzyme. Caspase-6 cleaves nuclear mitotic apparatus protein (NuMA) and mediates the shrinkage and fragmentation of cell nuclei. Caspase-8 is an initiation caspase that activates the caspase cascade during apoptosis, while caspase-9 is the initiator caspase in the caspase cascade in apoptotic normal and neoplastically transformed cells. During our immunocytochemical study, a sensitive, four-step, alkaline phosphatase conjugated antigen detection technique was employed. The results did in fact demonstrate the presence of high apoptotic activity within the cellular microenvironment of high-grade astrocytomas and glioblastomas. The observations identified cytoplasmic expression of caspase-3 and caspase-6 in more than 50 per cent of tumor cells, caspase-8 and caspase-9 in more than 10 per cent of tumor cells in high-grade anaplastic ASTR and glioblastoma. The immunocytochemical expression pattern in about 10 per cent of the tumor cells for caspase-3 and caspase-6 and about 1 to 5 per cent of the tumor cells for caspase-8 and caspase-9 demonstrated a translocation tendency from the cytoplasm to the cell nuclei in the apoptotic cells. This phenomenon may play an important role in these tumors' maintenance of immune privilege and evasion of immune attacks. We suggest that caspase-3, -6, -8 and -9 immunocytochemistry could have prognostic and immunotherapeutic significance in the treatment of these highly malignant glial tumors.

Apoposis or programmed cell death (PCD)

"Apoptosis is a cell suicide mechanism that enables metazoans to control cell number in tissues and to eliminate individual cells that threaten the animal's survival. Certain cells have unique sensors, termed death receptors, on their surface. Death receptors detect the presence of extracellular death signals and, in response, they rapidly initiate the cell's intrinsic apoptosis machinery" (1).

Normal development (pre- and postnatal), cell maturation and differentiation in a multicellular mammalian organism, as well as the maintenance of cellular homeostasis, require a perfect, dynamic co-regulation of cell proliferation and cell death (2,3). Furthermore, the balance between cell proliferation and cell death determines the growth and differentiation of every complex multicellular tissue. Three decades ago, Kerr and co-authors proposed the introduction of the scientific term "apoptosis" from the Greek apo (away from) and ptosis (falling) for the morphological nomenclature (4). Apoptosis is thus described as a process of leaves falling from the trees or the shedding of petals from flowers. It is a form of suicidal cell death that requires the active cellular synthesis of molecules involved in processes that provide the energy necessary for programmed cell death (PCD) to occur. PCD is a distinct mode of cellular suicide and represents one of the most important regulatory mechanisms of homeostasis, because it eliminates abundant and unwanted cells (5).
Specifically, apoptosis is a physiological process wherein the cell initiates a sequence of events culminating in the fragmentation of its DNA, nuclear collapse and, finally, disintegration of the cell into small, membrane-bound apoptotic bodies. Dramatic changes in the morphological structure of cells such as: 1) condensation of the nuclear (chromatin) and cytoplasmic structures (especially the mitochondria); 2) blebbing of the cell membrane; 3) characteristic swelling of the endoplasmic reticulum; and 4) fragmentation of the cells in membrane-bound apoptotic bodies, are the signs of total cell destruction. Complete breakdown of the cell membrane and cytoplasm is associated with the advanced stage of apoptotic changes, during secondary necrosis and this can be identified only in in vitro experiments (6,7).

The family of cysteine aspartyl proteases or caspases. Caspases are the executioners in the apoptotic or programmed cell death (PCD) pathway (8). Molecular biological research revealed that the caspase family functions as a well-directed orchestra of at least 14 proteases, all containing a common active site of cysteine within the conserved peptide sequence QACXG. Their cleavage target is the peptide bond C-terminal to aspartate residues. Caspases are divided into three subgroups upon their tetrapeptide sequence recognition: Group I caspases (caspase-1, caspase-4 and caspase-5) generally prefer the (W/L)EHD sequence; Group II caspases (caspase-3 and caspase-7) prefer DEXD; and Group III caspases (caspase-6, caspase-8, caspase-9 and caspase-10) recognize (L/V)EXD. The VAD tripeptide is targeted by all caspases; therefore, VAD is the structural base for pan-caspase reagents (9).

All caspases are present in mammalian cells in the form of enzymatically inertzymogens (named procaspases). Thezymogens undergo cleavage, resulting in four large and small subunits. These active or mature caspases represent a functional tetramer containing two distinguished active sites and two heterodimer subunits, one 20 kD (p20) and an other 10 kD (p10) (10). The prodomain is typically short for the so-called effector caspases (caspase-3 and caspase-7) and longer for the rest of them. The longer prodomain can include recognizable motifs, such as the CARD (caspase activation and recruitment domain: in caspase-1, caspase-2, caspase-4 and caspase-9) and the DED (death effector domain: in caspase-8 and caspase-10).

Recently, it has been shown that caspases target more than 100 various proteins belonging to diverse groups, such as signaling, regulatory, structural, etc. The activities of caspase family members as part of the complex PCD process are regulated by a number of complex interactions between them, adapter proteins and target substrates and different types of receptor families (11-13).

The intrinsic part of the PCD program arises from the mitochondria or the so-called 'killer organelles' (14), because it is the release of cytochrome c from the mitochondrial intermembrane space into the cytosol that develops into the caspase-activating complex or apoptosome (15). The apoptosome is completed from cytochrome c, ATP, Apaf-1 and caspase-9zymogen, the last two of which interact via their CARDs. Smac (or DIABLO) is a recently identified, novel proapoptotic molecule that is also released from mitochondria into the cytosol during apoptosis. Smac functions by eliminating the caspase-inhibitory properties of the inhibitors of apoptosis proteins (IAP), particularly XIAP (16). After the initial caspase-9 activation and the activation of the following effector caspases, the so-called terminal caspase-cascade is built up, a two-step process that is essential to PCD. The high level of PCD is ensured after autocatalytic activation, processing a number of caspases and regulatory proteins, which strongly amplify the apoptotic process.

Caspase-3. Caspase-3 is ubiquitously expressed in human cells and, like other caspases, is synthesized as an inactive, 32 kDa proenzyme. Upon activation, caspase-3 is cleaved at Asp28-Ser29 and Asp175-Ser176, thereby generating two subunits of 17 kDa and 12 kDa, respectively. Activation of caspase-3 occurs in response to a wide variety of PCD inducers including Fas-FasR. The in vivo patterns of CPP32 (caspase-3) gene expression were determined by Krajewska and co-workers employing an immunohistochemical approach and formalin-fixed, paraffin wax-embedded normal human tissues (17). Krajewska and co-workers used a rabbit polyclonal antiserum against recombinant human CPP32 protein, which was proven specific by immunoblot analysis of various human tissues and cell lines. CPP32 immunoreactivity was selectively found in certain cell types and was typically present within the cytosol, although occasional cells also contained nuclear immunostaining. CPP32-positive normal cells included epidermal keratinocytes, cartilage chondrocytes, bone osteocytes, heart myocardioocytes, vascular smooth muscle cells, bronchial epithelium, hepatocytes, thymocytes, plasma cells, renal tubule epithelium, spermatogonia, prostatic secretory epithelial cells, uterine endometrium and myometrium, mammmary ductal epithelial cells and the gastrointestinal epithelium of the stomach, intestine and colon. In contrast, weak or absent CPP32 immunoreactivity was observed in endothelial cells, alveolar pneumocytes, kidney glomeruli, mammmary myoepithelial cells, Schwann cells and most types of brain and spinal cord neurons. Consistent with a role for CPP32 in apoptotic cell death, clear differences in the relative intensity of CPP32 immunostaining were noted in some shorter-lived types of cells compared to longer-lived, including (a) germinal center (high) versus mantle zone (low) B lymphocytes within the secondary follicles of lymph nodes, spleen and tonsils; (b) mature neutrophils (high) versus myeloid progenitor cells (low) in bone marrow; (c) corpus luteal cells (high) versus follicular
granulosa cells (low) in the ovary; and (d) prostate secretory epithelial cells (high) versus basal cells (low). CPP32 was also found to be highly expressed in atherosclerotic plaques and to be colocalized with apoptotic cells (18).

According to the literature, immunocytochemistry of caspases has only been carried out in neuroblastosomas, but not in ASTRs. Frequently, neuroblastosomas demonstrate spontaneous regression and cellular differentiation, which may at least partly be regulated by signaling done through the nerve growth factor and its receptors, TRK-A and p75LNTR (19). Caspase-3 is actually required for the activation of four other caspases (-2, -6, -8 and -10) and also participates in a feedback amplification loop involving caspase-9 (23).

Caspase-6. The observation that the nematode cell death effector gene product Ced-3 is homologous to human interleukin-1beta-converting enzyme (caspase-1) has led to the discovery of at least nine other human caspases, many of which are implicated as mediators of apoptosis (20-22). The activity of ICE-like proteases or caspases is essential for apoptosis (23). Multiple caspases participate in apoptosis in mammalian cells but how many caspases are involved and what their relative contribution to cell death is poorly understood. To identify caspases activated in apoptotic cells, an approach to simultaneously detect multiple active caspases was developed. Employing neoplastically transformed cells as a model, it has been established that CPP32 (caspase-3) and Mch2 (caspase-6) are the major active caspases in cells undergoing PCD, being activated in response to distinct apoptosis-inducing stimuli and in all cell lines analyzed. Both CPP32 and Mch2 are present in apoptotic cells as multiple active species. In a given cell line, these species remained the same irrespective of the apoptotic stimulus used. However, the species of CPP32 and Mch2 detected varied between cell lines, indicating differences in caspase processing. The influence of a number of environmental parameters, including pH, ionic strength, detergent and specific ion concentrations, on the activity and stability of four caspases involved in death receptor-mediated apoptosis have been observed (22).

As already alluded to, caspases play a major role in the transduction of extracellular apoptotic signals and execution of PCD in mammalian cells (24). Ectopic overexpression of the short prodomain caspase-3 and -6 precursors in mammalian cells does not induce apoptosis, which is due to their inability to undergo autocatalytic processing/activation, suggesting that they depend on the long prodomain caspases for activation. Since caspase-3 and -6 are the most downstream executors of apoptosis, the constitutively active versions of these caspases could be used at very low concentrations in gene therapy model systems to induce apoptosis in target tissues or neoplastically transformed cells.

Caspase-8 (Mch5). The Fas/APO-1-receptor-associated cysteine protease, caspase-8 or Mch5 (MACH/FLICE), is believed to be the enzyme responsible for activating a protease cascade after Fas-receptor ligation, which leads to cell death (25). Binding of caspase-8 to the Fas receptor results in oligimerization of the caspase-8 protein, which in turn drives its autoactivation through self-cleavage. Once activated, the caspase-8 activates the downstream caspases, committing the cell to undergo PCD. The Fas-apoptotic pathway is potently inhibited by the cowpox serpin CrmA, suggesting that Mch5 could be the target of this serpin. Bacterial expression of proMch5 generated a mature enzyme composed of two subunits, which are derived from the precursor proenzyme by processing at Asp-227, Asp-233, Asp-391 and Asp-401. Recombinant Mch5 is able to process/activate all known ICE/Ced-3-like cysteine proteases and is potently inhibited by CrmA. This contrasts with the observation that Mch4, the second FADD-related cysteine protease that is also able to process/activate all known ICE/Ced-3-like cysteine proteases, is poorly inhibited by CrmA. These data suggest that Mch5 is the most upstream protease that receives the activation signal from the Fas-receptor to initiate the apoptotic protease cascade that leads to activation of ICE-like proteases (TX, ICE and ICE-reI).33, Ced-3-like proteases (CPP32, Mch2, Mch3, Mch4 and Mch6) and the ICH-1 protease. On the other hand, Mch4 could be a second upstream protease that is responsible for activation of the same protease cascade in CrmA-insensitive apoptotic pathways.

Tumor necrosis factor alpha (TNF-alpha) binding to the TNF receptor (TNFR) potentially initiates apoptosis and activates the transcription factor nuclear factor kappa B (NF-kappaB), which suppresses PCD by an unknown mechanism (26). The activation of NF-kappaB was found to block the activation of caspase-8. The induction of apoptosis could, in fact, be prevented by treating cells with an inhibitor of caspase-8, implying a direct relationship between caspase-8 and apoptosis (27).

Caspase-9. Caspase-9 was initially purified and identified as the third protein factor, Apaf-3, that participates in caspase-3 activation in vitro. Procaspase-9 and Apaf-1 bind to each other via their respective NH2-terminal, CED-3 homologous domains in the presence of cytochrome c and dATP, an event that leads to caspase-9 activation.Activation of procaspase-9 by Apaf-1 in the cytochrome c / dATP-dependent pathway requires proteolytic cleavage to produce the mature caspase-9 molecule (28). On the other hand, deletion of Apaf-1’s WD-40 repeats leaves Apaf-1 constitutively active and capable of processing procaspase-9 independently of cytochrome c and dATP (29). Apaf-1-mediated processing of procaspase-9 occurs at Asp-315 by an intrinsic autocatalytic activity of procaspase-9 itself (30).
that caspase-9 is a critical upstream activator of the caspase

glial tumors are characterized by a high tendency to local

hallmark for histopathological diagnosis (38). Furthermore,

effective tissue capable of inducing neoangiogenesis (36,37).

aggressive neoplasm that is regarded as the prototype of

glia (nerve cement).” - Rudolf Virchow (32)

This peculiarity of the membrane, namely, that it becomes continuous with the interstitial matter, the real cement, which links the nervous elements together and that in all its properties it constitutes a tissue different from the other forms of connective tissue, has induced me to give it a new name, that of neuro-glia (nerve cement).” - Rudolf Virchow (32)

Malignant childhood ASTRs represent neuroectodermal tumors appearing within the neuro-glial or macroglial central nervous system (CNS) (33). Gliomas can grow anywhere in the CNS, but in children they usually occur in the brain stem, the cerebrum, or the cerebellum. The most common brain tumors develop from glial cell precursors (astrocytes, oligodendrocytes and ependymocytes). Astrocytomas thus account for about 68 percent of the primary brain tumors occurring in children younger than age 20 (34,35).

GBM, a grade IV astrocytoma in the World Health Organization (WHO) classification, is a highly malignant and aggressive neoplasm that is regarded as the prototype of neoplastic tissue capable of inducing neoangiogenesis (36,37). The vascular phenotype of this tumor type represents a hallmark for histopathological diagnosis (38). Furthermore, glial tumors are characterized by a high tendency to local invasiveness and a relatively low metastatic potential. Proteolytic enzymes, such as the cysteine proteinase cathepsin S (CatS), have been implicated in the local invasion by neoplastically transformed astrocytoma cells resulting in tumor recurrence even after the surgical resection (39). Flannery and co-workers’ immunohistochemical study revealed that the highest levels of CatS activity were present in grade IV tumors.

The specific aims of this study were: 1) to identify the expression of caspase-3, -6, -8 and -9 in neoplastically transformed astrocytic cells; 2) to identify the presence of caspase-3, -6, -8 and -9 in TIL, which thereby destroys the host’s anti-neoplastic tumor-infiltrating effector cells; and 3) to identify the intensity of apoptotic cell death within high-grade ASTRs and glioblastomas.

**Materials and Methods**

**Tissues and tissue handling.** In this immunohistochemical study, we employed formalin-fixed, paraffin-wax-embedded tissue sections of human primary childhood anaplastic ASTRs (DAKO Corporation, Carpinteria, CA, USA). The diagnoses of the specific subtypes were established according to WHO guidelines for the classification of glioma by a clinical neurohistopathologist (40-44). Technical details of the immunohistochemical techniques used in this study have already been elaborated by other investigators (45-47) and in the studies published by our group (34,48-52).

**Monoclonal antibodies**

- Anti-caspase-3 mouse monoclonal antibody was obtained from Labvision (Fremont, CA 94539, USA; Cat. # MS-1121). Recombinant full-length human caspase-3 protein was employed as immunogen. Isotype: IgG2a. Clone: 3CSPO1, same as 7.1.44. The MoAB was purified from the ascites fluid by Protein A chromatography.
- Anti-caspase-6 mouse, anti-human monoclonal antibody (Cat. # MS-1126; Labvision). Recombinant full-length human caspase-6 prodomain (34 kDa) was employed as immunogen. Ig Isotype/Light chain: IgG1/k. Clone: 6CSP03, same as 14.1.190. The MoAB was purified from the ascites fluid by Protein G chromatography.
- Anti-caspase-8 mouse monoclonal antibody (Cat. # MS-1143-R7, Labvision). Recombinant full length human caspase-8 protein was employed as immunogen (mol. weight of antigen: 55 kDa). Ig Isotype: IgG1. Clone: 8 CSP03 (same as 4.1.20). The MoAB was purified from the ascites fluid by Protein A chromatography. Immunoreactivity: cytoplasmic.
- Anti-caspase-9 mouse monoclonal antibody (Cat. # MS-1146-P2; Labvision). Recombinant full length human caspase-9 protein was employed as immunogen (mol. weight of antigen: 46-48 kDa). Ig Isotype/light chain: IgG1/k. Clone: 9 CSP03 (same as 96.1.23). The MoAB was purified from the ascites fluid by Protein A chromatography. Immunoreactivity: cytoplasmic. Staining of formalin-fixed and paraffin wax-embedded tissue sections requires an antigen retrieval technique, employing preliminary boiling in 10mM citrate buffer, pH 6.0 (NeoMarkers, Cat. # AP-9003) for 10-20 minutes followed by cooling at room temperature for 20 minutes.
Figure 1. Childhood anaplastic ASTR. Expression of caspase-3 in the cytoplasm of neoplastically transformed cells. Routine, 10% buffered formalin fixation. Paraffin-wax embedding. Alkaline phosphatase conjugated streptavidin-biotin antigen detection technique. Magnification: 200x.

Figure 2. Childhood glioblastoma. Presence of caspase-8 in the cytoplasm and some nuclei of neoplastic cells. Routine, 10% buffered formalin fixation. Paraffin-wax embedding. Alkaline phosphatase conjugated streptavidin-biotin antigen detection technique. Magnification: 200x.
**Antigen retrieval.** No single antigen retrieval solution works well with all antigenic epitopes and as such, directions from the antibody manufacturer should be followed. In this immunocytochemical research project, we employed the immunohistochemical method of “antigen liberation” or “antigen retrieval”, when necessary (53-56). In the first step, antigen retrieval was sometimes achieved by single or combined enzymatic digestion (ficin, pepsin and trypsin from Zymed Labs., South San Francisco, CA, USA) prior to the primary antigen-antibody reaction. Heat-induced epitope retrieval (HIER) (57,58), as modified by us, was also employed. Antigen retrieval required immersion of tissue sections in a Target Retrieval Solution (DAKO Corporation) and heating in a water bath (95°C to 99°C). Unmasking of fixed antigen epitopes was also carried out by a single or combined enzymatic digestion prior to the primary AA reaction. An increase in the quantity of detectable antigenic epitopes following HIER has been described for a number of antibodies. Our method using citrate-buffer (NeoMarkers, Union City, CA 94587, USA; Cat.# AP-9003) solution worked well, employing preliminary boiling in 10mM citrate buffer, pH 6.0 for 10-20 minutes followed by cooling at room temperature for 20 minutes. Immunomorphological observations reported a significant increase in the intensity of immunostaining in response to this antigen retrieval technique.

**Immunohistochemical controls.** In order to ensure the specificity of the anti-caspase-3, -6, -8 and -9 antibodies used in this study, we tested the immunoreactivity of several normal human control tissues including: brain, adrenal, heart, stomach, small intestine, large intestine, liver, kidney, pancreas, lung, testis, ovary, uterus, prostate, thyroid and spleen, all included in one checkerboard multitissue block (DAKO Corporation; code # T1065) (59,60). Several postnatal human thymic specimens were also used as negative and positive tissue controls. A number of neoplastically transformed tissues, including malignant melanoma and lung cancer tissues, represented the positive tissue controls. Additional controls for all tissues and MoABs included: 1) omission of the primary, anti-caspase-3 MoAB; 2) utilization of only the enzymatic developer solution to detect the presence of endogenous alkaline phosphatase activity; and 3) utilization of MOPC 21 mouse myeloma IgG1 (ICN) as a replacement for the primary MoAB to determine non-specific myeloma protein binding to the antigen epitopes of the screened tissues.

**Immunohistochemical evaluation.** Qualitative and quantitative evaluation of the percent of antigen-positive cells and the intensity of caspase-3, -6, -8 and -9 immunostaining were conducted using a light microscope (Olympus, Japan) counting 100-200 cells from each of five to eight distinct areas in non-necrotic, ASTR, melanoma, lung adenocarcinoma and postnatal thymus tissues. Artifacts were avoided, while, on the other hand, morphologically characteristic areas were sought out. The presence of neoplastically transformed astrocytes and oligodendrocytes with heterogeneous IPs, the endothelial elements of small blood vessels, tumor infiltrating leukocytes and macrophages (the host’s immunological effector cells) required careful qualitative assessment. Non-vascular elements were also examined, but only morphologically distinct ASTR cells were scored.

Quantitative evaluation (61): (+ + + +) over 90% of the total cell number are positive; (+ + +) 50 to 90% of the total cell number are positive; (+ +) 10 to 50% of the total cell number are positive; (+) 1 to 10% of the total cell number are positive; (±) under 1% of the total cell number are positive; (−) negative. Qualitative evaluation (61): (A) very intense red staining; (B) strong red staining; (C) light red staining; (D) negative staining.

**Results**

In this immunohistochemical study, the presence and cellular localization of caspase-3, -6, -8 and -9 were observed in anaplastic, high-grade ASTRs and in GBMs, employing a sensitive, four-step, alkaline phosphatase conjugated antigen detection technique. The immunoreactivity demonstrated a dominant cytoplasmic pattern in more than 50 per cent of the tumor cells with high intensity staining (+ + +, A,B) for caspase-3 and caspase-6 while a dominant cytoplasmic pattern of caspase-8 and -9 was found in more than 10 per cent of the astrocytic brain tumor cells with high intensity staining (+ +, A,B). In about 10 per cent of the neoplastic cells, caspase-3 (CPP32) (Figure 1) and caspase-6, and in about 1 to 5 per cent of the neoplastic cells, caspase-8 (Figure 2) and -9, were translocated from the cytoplasm into the nuclei in regressing, apoptotic cells. A similar antigen expression pattern was observed in TILs. The presence of active or mature caspase-8 and -9 in the tumor infiltrating TIL cells is indicative of a possible escape mechanism favorable for the further tumor development and local invasion.

**Discussion**

Our immunohistochemical study identified cytoplasmic expression of caspase-3 and -6 in more than 50 per cent of tumor cells and caspase-8 and -9 in more than 10 per cent of tumor cells in high-grade anaplastic ASTR and GBM. The presence of caspases -3, -6, -8 and -9 were also detected in the TILs, representing the host’s immune effector, mostly cytotoxic cells.

The immunohistochemical expression of caspases during disease progression was published in a variety of neoplastically transformed cells. For example, the presence of caspase -3, -6 and -8 and their association to PCD was observed in neo-neoplastic and neoplastic lesions of the breast (62). The observed material consisted of 9 benign breast epithelial hyperplasias, 15 atypical hyperplasias, 74 breast (62). The observed material consisted of 9 benign breast epithelial hyperplasias, 15 atypical hyperplasias, 74 breast ductal epithelium, was seen in 22% of benign epithelial hyperplasias, 25% of atypical hyperplasias, 58% of in situ carcinomas and 90% of invasive carcinomas (dural or lobular or both). The corresponding percentages for caspase -6 and -8 were 11%, 25%, 60%, 87% and 22%, 57%, 84%, 83%, respectively. In high-grade in situ lesions, there were significantly more cases with strong caspase -3, -6 and -8 immunoreactivity than in low- and intermediate-grade lesions (p=0.0045, p=0.049 and p=0.0001, respectively). In invasive carcinomas, however, no
association between a high tumor grade and caspase -3, -6 or -8 expression was found (p = 0.27, p = 0.26 and p = 0.69, respectively). The mean apoptotic index was 0.14±0.14% in benign epithelial hyperplasias, 0.17±0.12% in atypical hyperplasias, 0.61±0.88% in in situ carcinomas and 0.94±1.21% in invasive carcinomas. In all cases, strong caspase -3, -6 and -8 positivity was significantly associated with the extent of apoptosis (p < 0.001, p = 0.015 and p = 0.050, respectively). The results demonstrated that synthesis of caspase -3, -6 and -8 is up-regulated in neoplastic breast epithelial cells in parallel to the increase in the apoptotic index and progression of the breast lesions.

It is well established that caspase -8 and -10 act as initiator caspases of the extrinsic apoptosis pathway, with caspase-9 as an initiator caspase of the intrinsic apoptosis pathway (63). Caspase-3 is considered to be the main effecter caspase involved in both intrinsic and extrinsic pathways. Alteration of apoptosis is essential for cancer development. Thus, analysis of the expression status of caspases, the main executioners of apoptosis, in cancer tissues is needed for a sophisticated understanding of cancer biology. As such, we explored caspase expression in brain tumors with the hope that the results may be useful for future therapies. In a similar study, Yoo and co-authors analyzed the expression of caspase-3, -8, -9 and -10 in 60 advanced gastric adenocarcinomas by immunohistochemistry employing a tissue microarray approach. Immunopositivity was observed for caspase-3 in 57 (95%), caspase-8 in 56 (93%), caspase-9 in 54 (90%) and caspase-10 in 58 (97%) of the 60 cancers. While 46 adenocarcinomas (77%) expressed all of the caspases examined, 14 cancers (23%) showed loss of expression in one or more caspases examined. Normal gastric mucosal cells showed none to weak expression of caspases 3, 8, 9 and 10. Taken together, these results suggest that stomach cancer cells in vivo may need caspase expression for PCD. Also, higher expression of the caspases in stomach cancer cells than in normal gastric mucosal cells suggests that apoptosis in susceptible stomach cancer cells might be easily triggered, thereby producing selective pressure to make more apoptosis-resistant cells during tumor development. The same could hold true for brain tumors.

The great majority of malignant glial tumors are incurable with the current classical, three therapeutic modalities, including surgical resection, radiotherapy and chemotherapy (64). This may well be the direct result of the biological variability of these tumors, e.g. participation of possible multiple stem cell lines in the histogenesis, intrinsic and acquired multidrug resistance. It is well established that low-grade ASTRs have an intrinsic tendency for progressive IP de-differentiation toward higher grade, more aggressive ASTRs.

Cell death in the core of human brain tumors is triggered by hypoxia and lack of nutrients, but the mode of cell death, whether necrosis or apoptosis, is not clearly defined (65). To identify the role of apoptosis in brain tumor cell death, the authors observed macromolecular (RNA and protein) synthesis and activity in the central to peripheral region of benign brain tumors derived from five patients who had not previously received radiotherapy or chemotherapy. Normal brain tissue (NBT) served as the control. RT-PCR analysis of tumor tissues covering central to peripheral regions detected mRNA overexpression of the pro-apoptotic gene bax in malignant tumors, indicating a commitment to apoptosis. In the mitochondria-dependent death pathway, caspase-9 and caspase-3 were overexpressed in tumors. The increased caspase-3 activity cleaved poly(ADP-ribose) polymerase (PARP). Agarose gel electrophoresis detected a mixture of random and internucleosomal DNA fragmentation in malignant brain tumors. Overexpression of pro-apoptotic bax, up-regulation of calpain and caspase-3 and occurrence of internucleosomal DNA fragmentation were present, indicating that one mechanism of cell death in malignant brain tumors is apoptosis and that the enhancement of this process therapeutically may promote decreased tumor growth.

The goal of a recent study was to investigate whether apoptosis occurs in T lymphocytes that invade (TIL) gliomas expressing Fas ligand (FasL)-expressing GBMs and if its induction could be mediated by Fas (66). Apoptotic T lymphocytes were detected in GBMs through the detection of cell-type markers combined with active caspase-3 immunohistochemical analysis, a recently introduced apoptosis-specific in situ ligation assay, as well as by examining morphological criteria. Apoptotic T cells expressed Fas and were localized in the vicinity or in direct contact with FasL-expressing tumor cells. The T lymphocytes were undergoing apoptosis in spite of Bcl-2 expression. Expression of Bax was also detected in dying T cells, thus explaining the absence of the protective effect of Bcl-2 because Bax inhibits Bcl-2 death-repressor activity. The results strongly suggested that GBM cells that express FasL can induce apoptosis in invading immune cells. This phenomenon may play an important role in these tumors' maintenance of immune privilege and evasion from immune attacks. Awareness of this phenomenon could be helpful in the development of novel strategies for immunological treatment of malignant, high-grade ASTRs.

Macrophages play an important role in the regulation and fate of neoplastically transformed astrocytic cells (67). Although gliomas contain an abundance of macrophages, their role in the apoptosis of gliomas is not well known. Chen and co-workers, in an experimental protocol to treat glioma cells, collected stimulated macrophages with lipo-polysaccharide and culture supernatants of activated macrophages. The results demonstrated that molecules released from activated macrophages significantly increased the apoptosis of glioma cells via Fas/FasL and caspase-3 pathways. The level of soluble
Fas did not appear to be involved in the mechanism responsible for apoptosis seen in this study, as its level was barely detected in both experimental and control groups. Two cytokines, TNFalpha and IFN gamma, were significantly elevated in the supernatant obtained from the activated macrophages. Considering the important role of these two molecules in the induction of apoptosis mediated by the Fas/FasL system, the present data suggest that TNFalpha and IFN gamma were the main molecules to trigger the cascade of apoptotic reactions in glioma cells. Hence, the results indicated that molecules released from the activated macrophages provide significant signals to stimulate the expression of Fas/FasL and caspase-3, which function to induce apoptosis in glioma cells.

Annonaceous acetogenins are a group of potential anti-neoplastic agents isolated from Annonaceae plants (68). In a recent study, annonacin, a cytotoxic mono-tetrahydrofuran acetogenin was purified from the seeds of Annona reticulata and analyzed for its biological effects. It induced Bax expression, enhanced caspase-3 activity and caused apoptotic cell death in T24 bladder cancer cells. These results suggest that annonacin is potentially a promising anti-neoplastic compound. Enhancing caspase activity should be further explored as a new treatment option in the fight against cancer.

Proapoptotic gene transfer to promote death or to augment killing by DNA-damaging agents represents a promising strategy for anti-neoplastic therapy (69). The Clontech Company (Palo Alto, CA, USA) has constructed an adenoviral Tet-Off trade mark vector with tightly controlled expression of Bid (Ad-Bid). Employing the non-small cell lung cancer cell lines H460, H358 and A549, low-dose Ad-Bid was shown to induce high levels of full-length Bid as well as caspase-3 and caspase-9 activity. Ad-Bid gene transfer resulted in mitochondrial changes consistent with PCD (mitochondrial depolarization, cytochrome c release), DNA fragmentation and a dramatic loss of cell viability. The proapoptotic effects of Ad-Bid were independent of p53 status and were augmented markedly by caspase-8 activators such as the DNA-damaging agent cisplatin. When Ad-Bid and cisplatin were used together, chemosensitivity was restored in p53-null H358 cells, increasing death from 35% following treatment with cisplatin and Ad-LacZ to >90% death with Ad-Bid and cisplatin (Ad-Bid alone induced 50% cell death under these conditions). Ad-Bid can induce PCD in neoplastically transformed cells and enhance chemosensitivity in the absence of p53, suggesting this approach as a high potential anti-neoplastic therapy.

Provoking PCD of glioma cells may represent a promising, biological intervention for future tumor treatment (70). Macrophages are able to induce apoptosis in a number of neoplastic type cells, including glioma cells. It is well-established that PCD is executed on either a death receptor-dependent or independent pathway. Whether and how apoptosis of glioma cells induced by activated macrophages is involved in these two pathways simultaneously are not known. However, it is established that the alteration of molecules related to both death pathways led to PCD of glioma cells and the inhibition of xenograft glioma growth in mice. Apoptosis of glioma cells, induced by the activated macrophage, is executed by way of both death receptor-dependent and independent pathways and such an apoptosis-induced approach can effectively inhibit the growth of glioma in vivo. As such, caspases can prove to be highly beneficial in the development of future therapies that trigger neoplastically transformed cell apoptosis. The recent article by Shinoura and co-workers discusses the novel strategies to induce PCD in glioma cells by transduction with adenoviral vectors carrying a variety of apoptosis-related genes, including Fas ligand, Fas, FADD, caspase-8, p53, p33ING1, p73alpha, Bax, Apaf-1, caspase-9, IkappaBdN, caspase-3, Bel-2 and Bel-X(L) (71). The results suggested that adenoviral vector-mediated delivery of apoptosis-related genes other than p53 is a potentially useful gene therapy approach toward the treatment of human brain tumors.

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