Article

Arq Neuropsiquiatr 2010;68(4):603-607

Caspase-3 and Bcl-2 expression in glioblastoma

An immunohistochemical study

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ABSTRACT

The unfavorable prognosis of malignant gliomas can also be explained by the incomplete knowledge of their molecular pathways. Studies regarding the regulatory process of apoptosis in glioblastoma (GBM), the most common malignant glioma, are few, and better knowledge of the expression of pro and anti-apoptotic proteins could collaborate with the development of new treatments founded on molecular basis. The objective of this study was to evaluate by immunohistochemistry the expression of caspase-3 and Bcl-2 in 30 samples of GBMs. The expression of caspase-3 (mean 17.67%) was lower than Bcl-2 (mean 30.92%), a statistically significant result ($p<0.0001$), suggesting low apoptotic activity in these tumors. Other studies of proteins related to the intrinsic and extrinsic pathway of apoptosis are required to provide additional information of this mechanism in GBMs.

Key words: glioblastoma, apoptosis, immunohistochemistry.

Expressão de caspase-3 e Bcl-2 em glioblastomas: um estudo imunohistoquímico

RESUMO

O prognóstico desfavorável dos gliomas malignos também pode ser explicado pelo pouco conhecimento dos seus mecanismos moleculares. Estudos relacionados à regulação do processo de apoptose em glioblastoma (GBM), o glioma maligno mais comum, são poucos, e o melhor conhecimento da expressão de proteínas pró e anti-apoptóticas poderia colaborar com o desenvolvimento de novos tratamentos fundamentados sobre a base molecular. O objetivo deste estudo foi avaliar por imunohistoquímica, a expressão de caspase-3 e Bcl-2 em 30 amostras de GBM. A expressão de caspase-3 (média de 17,67%) foi menor que a de Bcl-2 (média de 30,92%), com resultado estatisticamente significante ($p<0.0001$), sugerindo menor atividade apoptótica nestes tumores. Outros estudos envolvendo proteínas relacionadas à via extrínseca e intrínseca da apoptose são necessários para fornecer informações complementares deste mecanismo em GBMs.

Palavras-chave: glioblastoma, apoptose, imunohistoquímica.

Astrocytic tumors are the most frequent forms of gliomas (65 to 70%). According to World Health Organization (WHO), these tumors are classified based on histological and clinical criteria in: pilocytic astrocytoma (Grade I), and diffusely infiltrative astrocytomas, diffuse astrocytoma (Grade II), the anaplastic astrocytoma (Grade III) and glioblastoma (Grade IV), the most malignant variety[1,2].

Glioblastoma (GBM) represents 29% of all brain tumors and 69% of astrocytic and

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Received 29 July 2009
Received in final form 21 December 2009
Accepted 4 January 2010

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oligodendroglial tumors. Despite surgery, chemotherapy and radiotherapy, most of GBMs have poor prognosis. The survival time is less than one year and the five year survival rate is less than 10%[3,4].

Apoptosis is a programmed cell death with significant role in development and homeostasis of multicellular organisms. Most of the biological processes during apoptosis are caused by cysteine proteases that are part of a large family of proteins known as caspases[5]. These proteins are homologous, and their enzymatic activity is divided into two classes: initiators and effectors[6].

The apoptotic response is mediated through two pathways: extrinsic and intrinsic, depending on the origin of the stimulus of death. The extrinsic pathway is initiated by the addition of extracellular ligands of death receptors on the cell surface death[6], while the intrinsic or mitochondrial pathway is triggered by the translocation of mitochondrial apoptotic Bcl-2 family members, such as Bax, Bad and Bim. The apoptotic pathways are still poorly known in GBMs[7]. The anti-apoptotic members of this family include BCL2 and BCLX preserve the integrity of mitochondria and prevent the release of Cytochrome C in the presence of apoptotic stimuli. The BCL2 gene encodes a protein of 26 kDa membrane-associated, which shows the program inhibit cell death and thus promote cell survival[8,9]. The caspases constitute a large family of proteins homologous with each other, enzymatic activity and that are activated in cells marked for death by apoptosis. They are the central components of apoptotic response and are generally divided into two classes: initiator and the effector caspases, wich includes caspase-3[6].

Studies related to the regulatory process of apoptosis in GBMs are few, and greater knowledge of the expression of these proteins is necessary to provide additional information to collaborate on new treatments planed on molecular basis in the near future

The aim of this study was to evaluate, by immunohistochemistry, the protein expression of caspase-3 and Bcl-2 in GBMs.

METHOD

This study comprised 30 samples of GBMs (13 women and 17 men), from 2002 to 2007, and was performed in the Faculty of Medicine of Ribeirão Preto, University of São Paulo. The study was approved by the Ethics Committee of the FMRP-USP.

The samples embedded in paraffin were cut in Reichert Jung 2040 microtome with 3µm thick, deparaffinized and hydrated. The sections were incubated with 3% H_2O_2 and Pierce solution to block endogenous peroxidase and biotin, respectively. Subsequently the antigen retrieval was performed in buffer 1 mM EDTA, pH 8.0 for caspase-3 and buffer 10 mM Tris Base, 1 mM EDTA, pH 9.0 for Bcl-2. The samples were incubated with primary antibodies against isoformas caspase-3 cleaved - (CPP32) monoclonal - Novocastra® in dilution of 1:200 (synthesized as an inactive 32 kD proenzyme and is processed during apoptosis to its active), and Bcl-2 - (NCL-BCL-2-486) monoclonal - Novocastra® in dilution 1:200 and with biotin-conjugated secondary anti-rabbit antibody (1:1000; Vector Laboratories Inc., Burlingame, CA) and streptavidin-conjugated peroxidase (Vecstatin Abc kit, Vector Laboratories Inc.). Color was developed by the addition of DAB (Sigma Chemical, St. Louis, MO). To evaluate the background reaction, procedures were also perfomed in sections incubated only with the secondary antibodies (indirect technique) or in the absence antibodies (direct technique). The number with positive staining for caspase-3 and Bcl-2 isoforms was measured by using a camera (Axio Cam, Zeiss, Germany) and the program Axiovision 4.6 (Zeiss, Germany), in an increase of 400×.

For the analysis of protein expression related to apoptosis (caspase-3 and Bcl-2), two fields in areas with higher concentration of positive cells ("hot spots" areas) were selected in each slide. This methodology was used because the protein marking was not always observed diffusely in the samples studied. In order to evaluate the staining intensity, we used a semi-quantitative method (+ to +++), and also calculated the percentage of stained (positive) cells in relation to the number of total cells.

Statistical analysis was performed using the Mann-Whitney test from the GraphPad Prism Version 4.00 for Windows (GraphPad Software, San Diego California USA). The p values<0.05 were considered statistically significant.

RESULTS

Protein expression of caspase-3 antibody

A semiquantitative analysis of caspase-3 showed that in most samples (22 - 73.33%) the protein expression was low (+), intermediate (++) in 5 samples (16.66%), and high (+++) in one sample (3.33%) and negative in two samples. The percentage of positive cells to caspase-3 ranged from 7.36% to 59.52% (Table and Fig 1).

Protein expression of Bcl-2 antibody

A semiquantitative analysis of Bcl-2 showed that the protein expression in most of the samples (18 - 60%) was intermediate (++), low (+) in 10 samples (33.33%) and high (+++) in two samples (6.66%). The percentage of positive cells for Bcl-2 ranged from 16.64% to 56.92% (Table and Fig 2).

The expression of the two proteins were different, the staining for caspase-3 antibody (mean 17.67± 2.043) was lower than Bcl-2 antibody (mean 30.92±1.954) (Fig 3), p<0.0001.

Table. Protein expression of caspase-3 and Bcl-2 in GBM represented by the percentage of positive cells and semi-quantitative analysis.

Samples	Bcl-2 % of positive cells	Bcl-2 Semi-quantitative analysis	Caspase-3 % of positive cells	Caspase-3 Semi-quantitative analysis
1	44.38%	++	18.02%	+
2	34.83%	++	–	–
3	30.93%	++	–	–
4	33.06%	++	15.23%	+
5	19.66%	+	15.08%	+
6	17.19%	+	37.02%	++
7	16.64%	+	59.52%	+++
8	52.36%	+++	7.36%	+
9	31.97%	++	25.11%	++
10	20.53%	+	14.08%	+
11	35.88%	++	11.45%	+
12	29.33%	++	12.73%	+
13	31.15%	++	14.55%	+
14	18.09%	+	21.28%	+
15	22.47%	+	24.48%	+
16	39.12%	++	14.74%	+
17	16.73%	+	26.39%	++
18	29.65%	++	15.38%	+
19	37.22%	++	20.17%	+
20	30.14%	++	19.05%	+
21	18.85%	+	25.15%	++
22	45.26%	++	9.12%	+
23	23.48%	+	24.76%	+
24	27.10%	++	12.04%	+
25	56.92%	+++	8.78%	+
26	38.81%	++	11.49%	+
27	44.72%	++	12.02%	+
28	21.63%	+	25.72%	++
29	26.70%	++	18.13%	+
30	32.95%	++	11.46%	+
Mean	30.92%		17.67%	

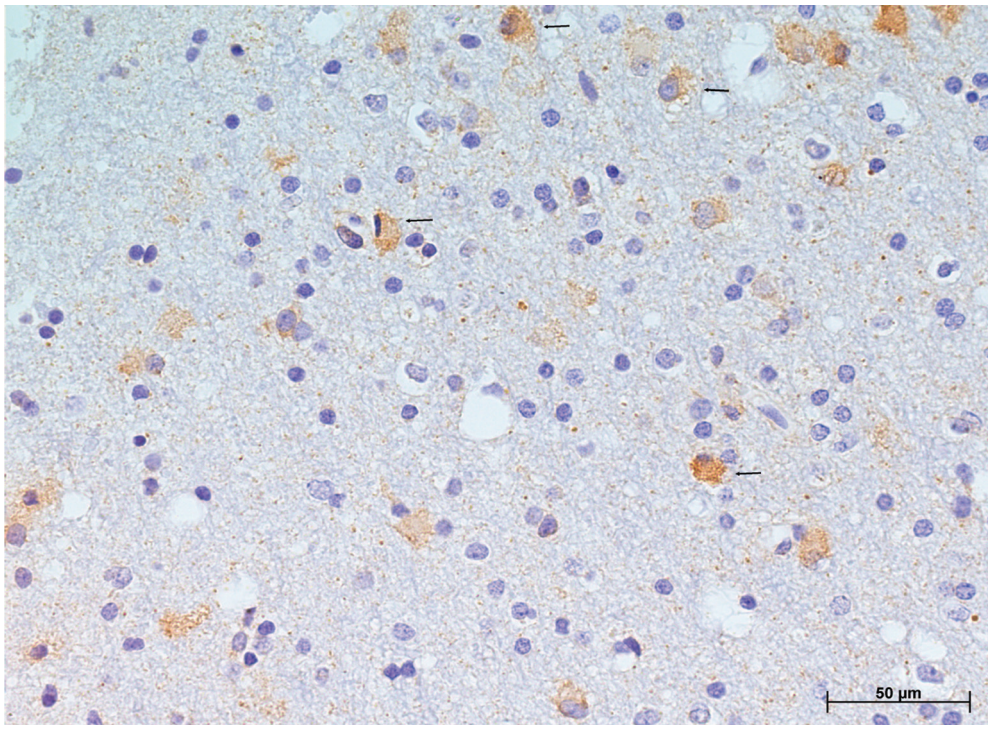

Fig 1. Photomicrography of caspase-3 expression in GBM. Positive cells are indicated by arrows (400×).

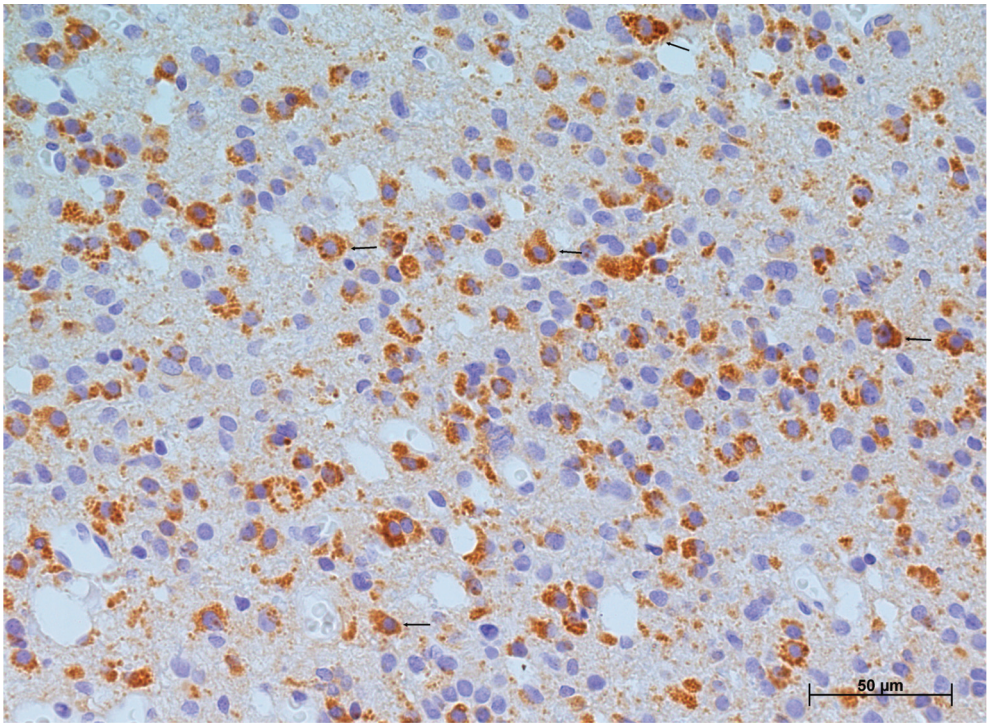

Fig 2. Photomicrography of Bcl-2 expression in GBM. Positive cells are indicated by arrows (400×).

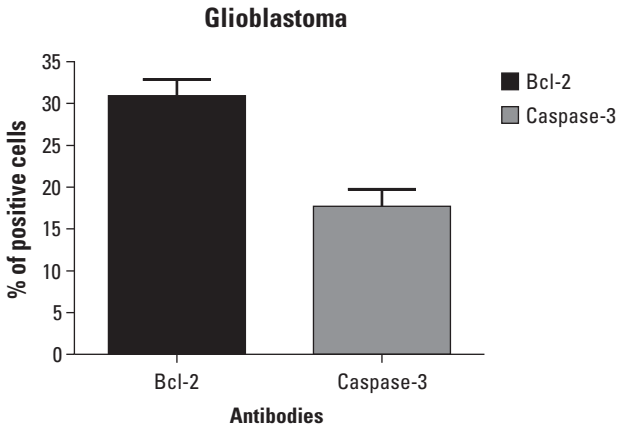

Fig 3. Mean and standard deviation of the percentage of positive cells to caspase-3 and Bcl-2 in GBMs.

DISCUSSION

Recent studies provide evidences that, contrary to the traditional view, most spontaneous cell deaths in malignant gliomas are due to apoptosis rather than necrosis[10].

Dysregulation of apoptotic mechanisms plays important role in the pathogenesis and progression of various cancers and also poor responses of tumors to therapeutic interventions. Highly invasive cancer cells are protected from apoptosis by up regulation of various anti-apoptotic molecules including B cell lymphoma-2 (Bcl-2) protein. Over expression of Bcl-2 provides a survival advantage to cancer cells in response to a wide range of apoptotic stimuli through inhibition of mitochondrial cytochrome c release[11,12]. Consistent with these findings, our results showed high protein expression of Bcl-2 (average of 30.92% of positive cells in 30 samples), providing molecular evidence that the protein Bcl-2 can reduce cell death in GBM.

George, Banik and Ray[13] studied the action of an anti-neoplastic drug, taxol, that binds b-tubulin, to prevent tumor cell division, promoting cell death. In this study, Bcl-2 expression was inactivated using siRNA during taxol treatment, in order to evaluate apoptosis in two GBM cell lines, U138MG and U251MG. Increased apoptosis occurred after treatment with taxol alone, Bcl-2 siRNA alone, and both agents together. Western blotting showed increased levels of Bax, tBid (pro-apoptotic members of the Bcl-2 family) and also active caspases that promote apoptosis. In summary, the result showed that chemotherapy could be more efficient if Bcl-2 could be inactivated.

Molecular events associated with apoptosis, including effectors caspases, such as caspase-3, has been shown to be present in the U87 GBM xenografts[14,15]. Zarnescu et al.[16] investigated the immunohistochemical expression of caspase-3, 9 and Bax in intracranial U87 GBM xenograft. The study showed that GBM xenografts contain positive cells for the three proteins. A previous immunohistochemical study indicated expression of caspase-3 in more than 50% of tumor cells and caspase-9 in more than 10% tumor cells from high-grade anaplastic astrocytomas

and GBMs[17]. Moreover, RT-PCR experiments and Western blot analysis have shown over expression of pro-apoptotic Bax and up regulation of caspase-3 and caspase-9 in GBM multiforme derived from patients who had not received previously radiotherapy or chemotherapy[18].

Recent studies offer a new perspective on presence of caspases, mainly caspase-3, in the tumor cells[19,20]. Gdynia et al.[20] reported that in the absence of cellular stress, active caspases are constitutively present in GBM cells and promote their motility. Moderate active caspase-3 levels are found in human GBM samples, freshly isolated GBM cells, and long-term cultured glioma cell lines. The amount of active caspase-3 and other caspases is not sufficient to induce apoptotic cell death, but it contributes substantially to the motility of GBM cells. Thus, the findings of Zarnescu et al.[16] reasonably speculate that the presence of immunostaining for caspase-3 in U87 GBM xenografts might be correlated both with migration in the host brain and apoptosis. In our study we observed lower protein expression of caspase-3 (average of 17.67% of positive cells in 30 samples) compared to the expression of Bcl-2, suggesting that anti-apoptotic mechanisms in these tumors are higher than the pro-apoptotics, although we do not have studied other effectors and initiators proteins related to intrinsic and extrinsic pathways of apoptosis. Nevertheless, seven samples showed higher expression of caspase-3 compared to Bcl-2, as 59.52% of positive cells in the sample seven. These results agree with those obtained by Zarnescu et al.[16] and as described, can be explained by the action of caspase-3 is involved in the process of cell migration.

The evaluation of the behavior of other apoptotic proteins and anti-apoptotic related to their intrinsic and extrinsic pathway are necessary for better understanding the cellular mechanism in GBM. Furthermore, cellular mechanisms such as angiogenesis, cell proliferation and cell adhesion, among others, as well as proteins related to the control of the cell cycle, may be involved as mechanisms in parallel characterization of the behavior of this type of tumor and may also explain the lower apoptotic index in our work.

In conclusion, this study presents evidence that apoptosis is inhibited in GBMs. Further analysis are need to be done to evaluate the therapeutic potential of apoptotic and anti-apoptotic proteins in the treatment of GBM.

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