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Signals of Apoptotic Pathways in Several Types of Meningioma

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Abstract Meningiomas are intracranial tumour derived from meningothelial cells, which aggressive behaviour has been frequently associated to cell apoptosis. In this paper activation of several factors involved in apoptosis has been investigated on biopsies of primary, non recurrent meningiomas. Benign (meningotheliomatous, transitional, fibrous, angiomatous), atypical and anaplastic meningiomas were analysed by immunohistochemistry and western blot, to visualize the occurring of different apoptotic pathways and their association with clinical grading. Apoptotic cell have been detected by a double colorimetric staining for TUNEL and caspase-3 active form. Apoptotic signal positive cells have been detected in all type of meningiomas analysed, with exception of meningotheliomatous meningiomas. Differences have been found in the activation of apoptotic pathways between several types of grade I meningiomas and among benign, anaplastic and atypical meningiomas. An intense expression of several apoptotic inhibitor occurred in grade I meningiomas. The correlation among expression of apoptotic and inhibitory factors and cell proliferation index may suggest that in grade I meningiomas apoptosis may be related to mechanisms involved into tumor cells surviving. Instead in grade II and III meningiomas the same correlation seems indicate an high turnover of tumor cells that might be useful as index of cell proliferation and tumor mass growth.

Keywords Caspases · Caspase-inhibitor · Bax · bcl-2 · TUNEL

Abbreviations

c-Flip	FLICE inhibitory protein
FLICE	FADD-like IL-1β-converting enzyme
SDS	Sodium Dodecyl Sulphate
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
XIAP	X-linked inhibitor apoptotic protein

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Introduction

Meningiomas are slow-growing intracranial tumors, derived from meningothelial cells. Meningiomas are classified histopathologically, in three categories: benign meningioma (further subdivided according to histological aspect in several types), atypical meningioma and anaplastic meningioma (malignant proper), or in accordance with their clinical behaviour respectively grade I, II and III of the WHO nomenclature [1, 2].

Apoptotic pathway activation is an important issue related to growth and clinical outcome of tumors. In fact a malfunction of the apoptotic pathways leads to the setting

of malignant tumors [3, 4]. Presence of apoptosis has been frequently associated to recurrence and/or aggressive behaviour of meningiomas [5, 6].

Apoptosis represent a programmed cell death, in this pathway caspases are executioner enzymes playing a fundamental role in degradation phase [7]. In many tumors cell transformation is associated with a genetic silencing or alteration of caspase activation process [8].

However the presence of caspase activation cannot be taken as an absolute signal for cell death, because caspase inhibitors have been found [9, 10]. Tumor cell can survive producing more caspase inhibitor factors such as survivin, that has been often associated to therapy resistance and poor prognosis in tumor clinical outcoming [5, 11]. The main downstream apoptotic executing caspases: the caspase-3, has been observed also to participate in proliferation/differentiation pathways [10]. Furthermore findings about an alternative programmed cell death characterized by bax-dependent/caspase-independent pathway have been observed leading authors to introduce the term "non-apoptotic cell death" pathway [12].

Apoptosis follows two different pathways: an intrinsic pathway caused by cellular oxidative/metabolic stress and mediated by disarrangement of the mitochondrion and caspase-9 activation, and an extrinsic pathway, triggered by binding of ligands to death receptors that in turn activate caspase-8 [13]. Both intrinsic and extrinsic pathways have in caspase-6 activation an important common point and increasing step [14]. Other factors such as bax pro-apoptotic protein and bcl-2 anti-apoptotic protein complete the biochemical intracellular pathways of apoptotic process [15, 16], that converge on activation of caspase-3 [17].

The most representative caspase inhibitor factors survivin and XIAP (X-linked inhibitor apoptotic protein), block apoptosis by interacting with caspase-9, -3 and -7 activated fragment [18], while another apoptotic inhibitor, c-FLIP (FLICE inhibitory protein) reduces the cleavage of caspase-8 and -9 [19]. Therefore it is difficult to assess the relation of caspases without to investigate a great number of factors.

In this contest a wide analysis of apoptotic pathways, their inhibitors and the cell proliferation index could be a valid tool to better identify the death/surviving pathway occurring in different meningioma types [20].

In this paper activation of several factors involved in apoptotic cell death and cell proliferation index has been investigated by immunohistochemistry and western blot. TUNEL technique was also used to assess DNA cleavage and cell death. Actually TUNEL technique is no more accepted as marker of pure apoptotic death, because its positivity has been seen related also to some necrotic or non-apoptotic death pathways [21, 22]. Therefore the presence of effective apoptosis in tumor mass has been assessed by a double stain for caspase-3 active fragment and TUNEL technique [23]. Analysis of leukocytes infiltrating tissue was added also to visualize their involvement in inducing tumoral cell apoptosis and their involvement in apoptotic signals detected [24].

Materials and Methods

Specimens

Fresh biopsy from totally resected intracranial meningiomas has been taken under study. Same exclusion criteria were adopted to obtain an homogeneous experimental group of meningiomas specimens such as: pharmacological treatment consisting in clinical anti-oedema and antiepileptic protocol treatment performed with cortisol 8 mg/kg and phenobarbital 100 mg/kg; lack of necrotic areas in grade I meningiomas; lack of bordering tissue infiltration that make less effective a complete tumor resection; biopsies from tumor at first operation, not recidival tumor mass.

95 meningiomas were evaluated in the present study (42 man and 53 woman; age: 63±12). In accordance with histopathological diagnosis and WHO criteria, they were differently classified as grade I meningiomas: 20 Meningotheliomatous (9 man and 11 woman), 20 Transitional (7 man and 13 woman), 18 Fibrous (8 man and 10 woman), 12 Angiomatous (5 man and 7 woman). Grade II meningiomas: 13 Atypical (6 man and 7 woman). Grade III meningiomas: 12 Anaplastic (5 man and 7 woman). Specimens of intact leptomeningis has been excised from some patients to use as control.

Tissue Processing

Each specimen coming from surgical excision of tumor, has been immediately washed in cold phosphate-buffered saline (PBS) and separated into two halves. One half was processed for Western blot as below detailed, the other one was rapidly fixed in 10% buffered formalin for 48 h at 4°C. These last specimens were then dehydrated and embedded in a semi-synthetic paraffin (Sherwood Medical co. St. Louis, Mo, U.S.A.). Then each specimen were cut serially with microtome (Leica-Jung, Germany) in consecutive section, 5 μm thick, and subdivided for morphological analysis in four groups of eight slides each (one section for slides).

The first slides of each groups were processed for Trichromic of Masson technique (DiaPath, Bergamo, Italy), to obtain histopathology visualization of tissue.

Immunohistochemistry

The second slides of each groups were processed to detect apoptotic cells by two-colour immunoperoxidase staining with TUNEL (Apoptag kit, Intergene company, Purchase, NY,

U.S.A.) and cleaved caspase-3 (rabbit polyclonal antibody, clone Asp-175; Cell Signaling Technology, Beverly, MA, U.S.A.) following protocol elsewhere described [25]. Briefly the TUNEL technique was performed, as elsewhere described [26], but the DAB reaction was intensify with nickel diaminobenzidine (Vectastaine Elite kit, Vector), yielding a black nuclear deposit onto TUNEL positive cell nuclei. Following slides were washed with in PBS and processed for caspase-3 immunohistochemistry as following detailed.

From the 3rd to 8th slides of each groups were processed respectively for immunohistochemistry of cleaved caspase-8 (mouse monoclonal antibody, clone Asp-384; Calbiochem, La Jolla, CA, U.S.A.), cleaved caspase-9 (rabbit polyclonal antibody, Ab-2; Oncogene, San Diego CA, U.S.A.), cleaved caspase-6 (rabbit polyclonal, clone H194; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), bax (rabbit polyclonal, clone P19; Santa Cruz Biotechnology), bcl-2 (rabbit polyclonal, clone ΔC21; Santa Cruz Biotechnology), Ki-67 (mouse monoclonal, Ab-1, Oncogene). Immunohistochemical technique was performed as following described.

The section were hydrated, and incubated in aspecific serum for 1 h at room temperature. Primary antibody incubation was performed overnight in a humid chamber at 4°C. Biotynilated anti-rabbit or anti-mouse secondary antibody and avidin/biotynilated enzyme complex were used, together with DAB chromogen, to visualize reaction (ABC Staining System kit, Santa Cruz). Hematoxylin was used to counterstain negative cell nuclei. Control sections were processed as above, but using a non-immune mouse IgG instead of primary antibody, or by omitting the primary antibody from incubation medium. In these conditions no specific immunostaining was observed.

Morphometric analysis was performed detecting positive cells profile following several immunoistochemistry and/or TUNEL technique by using an image analyser (Qwin, Leica). For each slide, five microscopic fields (area of investigation = 5×10^4 μm^2) were analysed.

Scored cells were collected and referred as % positive cells (determined by the ratio of positive cell/total cell number) detected on $5\times(5\times10^4)$ μm^2 field area.

Data reported as TUNEL positive nuclei (alone) was detected counting TUNEL positive nuclei on 2nd slides of each slide-group.

Leukocytes tissue invasion was morphologically detected and scored using trichromic staining slides (area of investigation as above detailed), leukocytes immunohistochemistry positivity was evaluated as well and reported as ratio of positive leukocytes/total leukocytes number scored on the same field of analysis

Western Blotting

An equal amount of protein of each sample obtained from homogenization and centrifugation of each meningioma, was loaded on a 12% SDS-polyacrylamide. Control specimens of normal leptomeninges were collected and homogenized.

The gel was transblotted to a nitrocellulose membrane for 1 h. Then membranes were treated with 3% bovine serum albumin in Tris-Tween buffer (PH 7.6) for 1 h. Primary antibody against both caspase proform and cleaved active fragment were used: caspase-8 (rabbit polyclonal, clone H134; Santa Cruz Biotechnology), caspase-9 (rabbit polyclonal, clone H170; Santa Cruz Biotechnology), caspase-3 (rabbit polyclonal, clone H277; Santa Cruz Biotechnology), caspase-6 (rabbit polyclonal, clone Ab-2, Oncogene). Furthermore antibody against bax (rabbit polyclonal, see above), bcl-2 (rabbit polyclonal, see above), c-FLIP (rabbit polyclonal; cat. 06-864; Up-State NY, USA), XIAP (mouse monoclonal, clone 2F1, Stressgen, Ann Arbor, Michigan U.S.A.), survivin (goat polyclonal, clone N18; Santa Cruz Biotechnology) were also used. The primary antibodies were detected by chemiluminescence substrate (ECL, Amersham International, Little Chalfont, U.K.) with horseradish peroxidase-coniugated anti rabbit, or anti-mouse or anti-goat IgG (Sigma, St. Luis, MI, U.S.A.).

By image analyser a semiquantitative densitometric analysis of specific band was performed in each western blot to evaluate apoptotic factors expression. For each caspase the expression of caspase precursor forms was evaluated summing densitometric values of the proform band and the active fragment band obtained in western blot analysis. To take in account optical density and extension of western blot band, each band was evaluated as *%grey scale index* × *area*$^{(-4)}$ *in pixel* (%OD), optical fundus value of each western blot panel was evaluated as covariate.

Data Analysis

Kolgomorov-Smirnov statistical test was used for assessment of the normal distribution of data. Means of different parameters investigated were calculated from single sample data, group means ± S.E.M., were then obtained from single samples values. Statistical analysis of variance (ANOVA) was used, the significance of differences between means was assessed by Newman-Keuls multiple range test, taking $p<0.05$ as the minimum level of significance.

Results

Common Features

Leukocytes have been observed to infiltrate meningiomas, leukocytes number was found increased in grade II and III meningiomas (data not shown).

In several meningiomas immunohistochemistry analysis showed 20% of total leukocytes positive for caspase-9

active fragment, bax and double colorimetric immunohistochemistry for caspase-3 active fragment and TUNEL (data not shown). Leukocytes, both immunopositive and not, were not associated to immunopositive meningioma cells (Fig. 1a).

No immunohistochemistry detection of caspase-6 active fragment positive cells were found in several meningiomas analysed.

Immunohistochemistry Analysis

Immunohistochemistry analysis of normal leptomeninges has shown rare cell immunopositive to caspase-8 active fragment (casp8-a.f.). No other positive signals have been detected.

Immunohistochemistry analysis of apoptotic signals in several meningiomas analysed were summarised in Figs. 2 and 3.

Immunohistochemistry analysis has shown casp8-a.f. positive cells in transitional and angiomatous meningiomas only, a greater number of casp8-a.f. positive cells were detected in transitional than in angiomatous meningiomas (Fig. 2). Casp9-a.f. positive cells were observed in transitional and fibrous meningiomas only among grade I meningiomas, while a lower number of casp9-a.f. positive cells were detected in grade II and III meningiomas. Casp3-a.f. positive cells were observed in all meningiomas analysed (Fig. 2). In grade I meningiomas we have detected a similar number of casp3-a.f. positive cells, with exception of meningotheliomatous ones in which a very large number of positive cells were detected (Fig. 1b); a lower number in casp3-a.f. positive cells were detected in grade II and III meningiomas in comparison with grade I meningiomas (Fig. 2). Bax and bcl-2 positive cells were observed in all meningiomas analysed. In particular comparing the several meningiomas analysed, we have detected the lowest number of bax positive cells in meningotheliomatous and fibrous meningiomas, the largest number of bax positive cells in transitional meningiomas, while angiomatous meningiomas (Fig. 1c) and grade II and grade III meningiomas showed a similar number of bax positive cells (Fig. 2). Instead a very low number of bcl-2 positive cells were detected in meningotheliomatous meningiomas only, while a large number of bcl-2 positive cells were detected in fibrous meningiomas. Transitional and angiomatous meningiomas together grade III meningiomas showed a similar number of bcl-2 positive cells, which were lower than those detected in fibrous meningiomas (Fig. 2).

In all meningiomas analysed with the exception of meningotheliomatous meningiomas TUNEL/caspase3 active fragment double (T+casp3-a.f.) positive cells were found (Figs. 1d and 2). The number of double positive cells were similar among several grade I meningiomas (Fig. 2). In grade

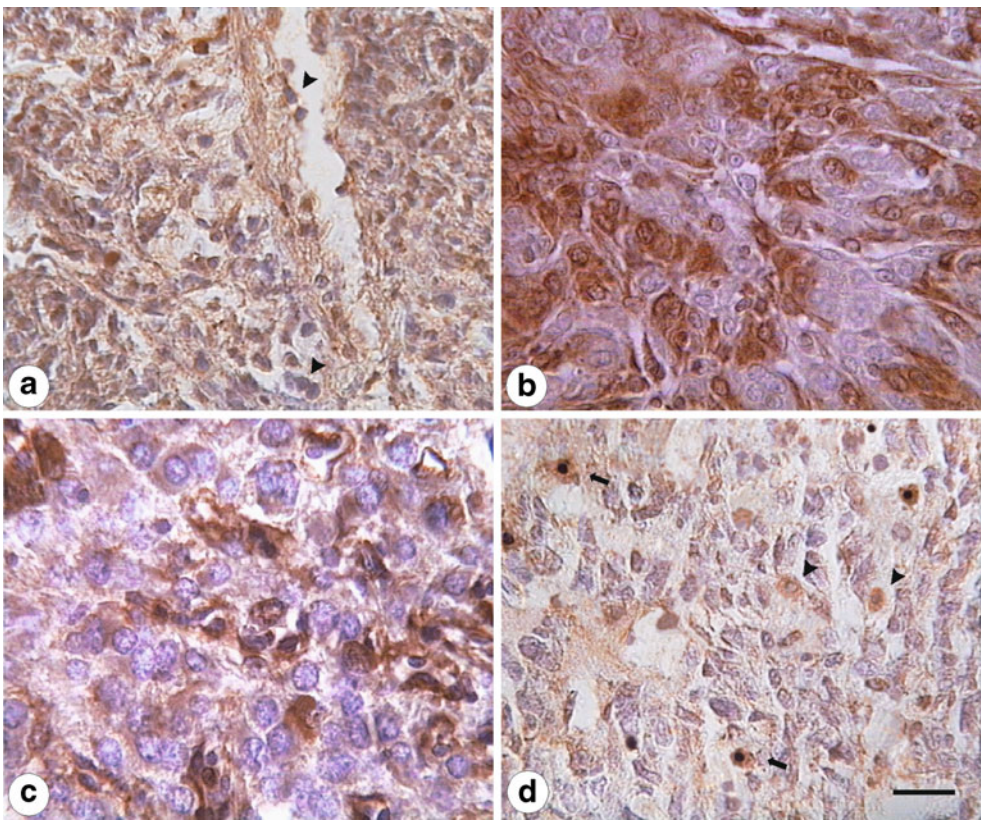

Fig. 1 Microphographs of apoptotic factors immunohistochemical staining. **a**: Leukocytes tissue infiltration, bax immunostaining in anaplastic meningioma (arrowheads= infiltrating leukocytes); **b**: caspase-3 immunostaining in meningotheliomatous meningioma; **c**: bax immunostaining in angiomatous meningioma; **d**: double immunohistochemistry TUNEL+Caspase-3 in anaplastic meningioma (*arrow*), arrowheads = caspase-3 positive cells. Calibration bar **a** and **d** = 60 μm; **b** and **c** = 30 μm

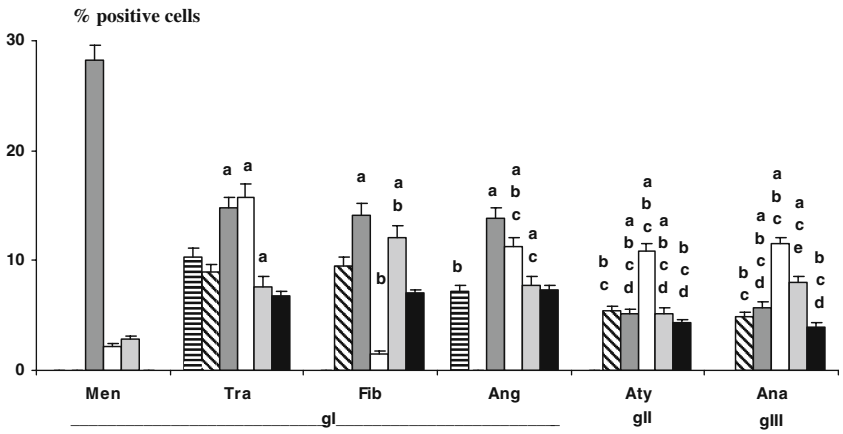

Fig. 2 Bar histogram showing the percentage of immunohistochemistry positive cell in several meningiomas studied. *Men* meningotheliomatous; *Tra* transitional; *Fib* fibromatous; *Ang* angyomatous; *Aty* atypical; *Ana* anaplastic. *Horizontal Band bar*: Caspase-8 immunohistochemistry; *Diagonal Band bar*: Caspase-9 immunohistochemistry; *Dark Grey bar*: Caspase-3 immunohistochemistry; *White bar*: Bax immunohistochemistry; *Light Grey bar*: bcl-2 immunohistochemistry; *Black bar*: double immunohistochemistry TUNEL+Caspase-3. *a*= $p<0.05$ vs. Men; *b*= $p<0.05$ vs. Tra; *c*= $p<0.05$ vs. Fib; *d*= $p<0.05$ vs. Ang; *e*= $p<0.05$ vs. Aty

II and III meningiomas a lower number of T+casp3-a.f. positive cells were detected (Fig. 2).

In meningotheliomatous meningiomas no TUNEL positive cells were found, (Figs. 1b and 3). Indeed in meningotheliomatous meningiomas, immunohistochemical positive cells were characterised by round cell nucleus profile with heterochromatic appearance, very different from characteristic cell nuclei apoptotic morphology (Fig. 1b).

In grade I meningiomas some casp3-a.f./not TUNEL positive cells were found, while a few number of these cells was observed in grade II and grade III meningiomas (Fig. 3).

TUNEL positive cells not co-expressing casp3-a.f. have been observed in grade II and III meningiomas only (Fig. 3).

Immunohistochemical analysis of Ki-67 positive cells have revealed an increase of positive cells in according to meningiomas malignancy grade. No significant differences among different types of grade I meningiomas were found in Ki-67 positive cell number (Fig. 3).

Western Blot Analysis

To verify the immunohistochemistry results, western blot analysis was performed, the results about apoptotic signals and inhibitor detected in several meningiomas analysed were summarized in Figs. 4 and 5. Western blot analysis on normal leptomeninges has not shown specific bands of caspase activation, nor bax, bcl-2 or inhibitors expression (data not shown).

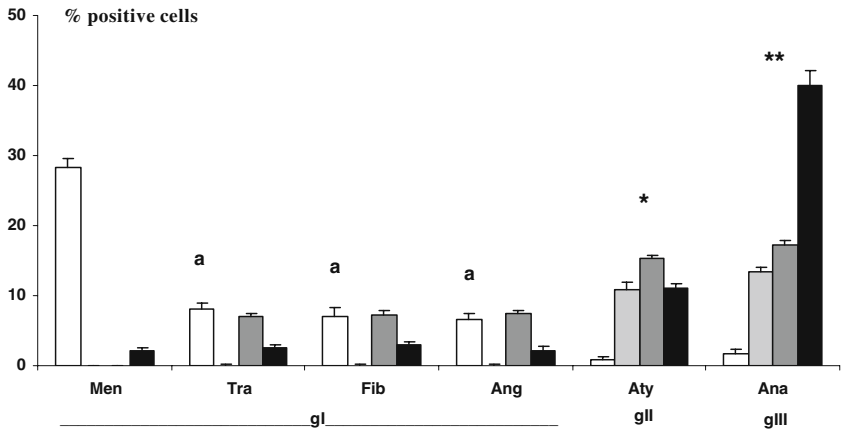

Fig. 3 Bar histogram showing the percentage of immunohistochemistry positive cell to TUNEL and caspase-3 active form. *White bar*: caspase-3 active form/no-TUNEL positive cells; *Light Grey bar*: no-caspase-3 active form/TUNEL positive cells; *Dark Grey bar*: Total TUNEL positive cells; *Black bar*: Ki-67 immunohistochemistry. *a*= $p<0.05$ vs. Men; *= $p<0.05$ vs. grade I meningiomas; **= $p<0.05$ vs. grade II and grade I meningiomas. Other legends like in Fig. 1

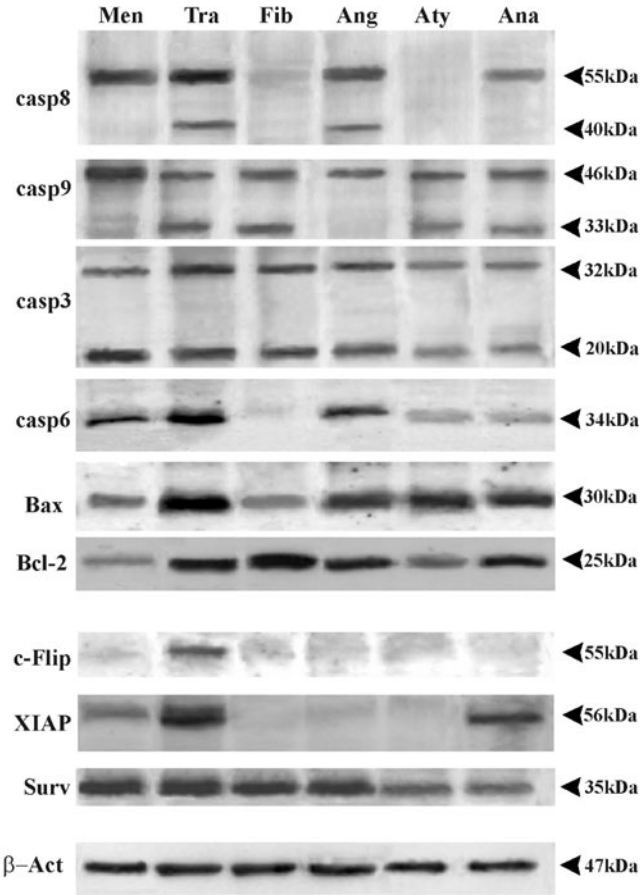

Fig. 4 Western blot analysis of expression and activation of apototic factors studied. casp= caspase, β-Act= beta-actin; Men, Tra, Fib, Ang, Aty, Ana= see legend in Fig. 1. In casp8, casp9, casp3 the double line indicates respectively caspase proform and active fragment as detected by primary antibody used. In casp6 only proform has been observed, no active fragments were detected

Western blot analysis in meningiomas has shown that the lack of caspase activation may be imputed to the lack of cleavage process, such as in meningotheliomatous and anaplastic meningiomas. In these meningiomas caspase-8 proform has been found expressed, but not its active fragment, instead in fibromatous and atypical meningiomas the lack of caspase-8 proform has been observed (Fig. 4).

The caspase-6 proform was expressed in all meningiomas analysed with exception of fibromatous ones, the lowest levels has been detected in grade II and grade III meningiomas. No activation of caspase-6 has been found in several meningioma types analysed (Fig. 4).

Western blot analysis of apoptotic step inhibitors has showed expression of c-Flip in transitional meningiomas only; XIAP in meningotheliomatous and transitional meningiomas and anaplastic meningiomas. Instead survivin expression was detected in all meningiomas analysed (Fig. 4).

Immunohistochemical data have been confirmed by densitometric analysis of caspase activation profile, bax and bcl-2 signals (Fig. 5). Summarising in atypical and anaplastic meningiomas we have found a lower level of caspase precursors and active forms expression compared to those found in grade I meningiomas (Fig. 5).

Densitometric analysis of apoptotic inhibitors showed higher level of XIAP expression in transitional meningiomas, and a very high expression of survivin was found in grade I meningiomas, while atypical and anaplastic meningiomas showed lower levels (data not shown).

Discussion

In meningioma samples analysed a higher number of TUNEL-positive cells and a different amount of apoptotic signals have been detected compared to that observed by other authors [6, 27]. Moreover a low amount of caspase-3 active form expression has been scored in grade II and III meningiomas. These differences observed might be due to the use of fresh specimen instead of archival ones [28, 29]. Otherwise a high expression of caspase-3 has been found associated to recurrence in meningioma [5, 6], but recurrence in the present work represent an exclusion criteria. Furthermore the samples taken in consideration in the present study came from patients subject to pharmacological treatment with cortisol: there are some indications that cortisol and similar agents such as dexamethasone may influence the expression of apoptotic signals in tumor cells [30]. We have not available a comparison with not treated patients and in literature no data about this topic has been found.

In our study, the presence of leukocytes into tumor tissue does not seem to influence directly the expression of apoptosis in tumor cells. Their low amount in term of cell number probably does not affect significantly the western blot signals. The apoptotic immunohistochemical signals detected into leukocytes are compatible with physiological performing of these cells that die for oxidative burst after activation [31].

Caspase-8 gene down regulation or alteration of its activation pathway (apoptotic intrinsic pathway) has been claimed as a primary findings in many tumour that make cells able to proliferate and survive [4, 32]. Indeed intrinsic apoptotic pathway activation (caspase-9 and bax) has been detected. However the strong presence of apoptotic inhibitor such as survivin XIAP, bcl-2 should make ineffective the intrinsic pathway. The presence of these apoptotic inhibitors do not alter caspase cleavage, but block their activity [11, 18].

Caspase-6 has been observed to represent an important increasing step in the apoptosis pathways [14]. In analysed

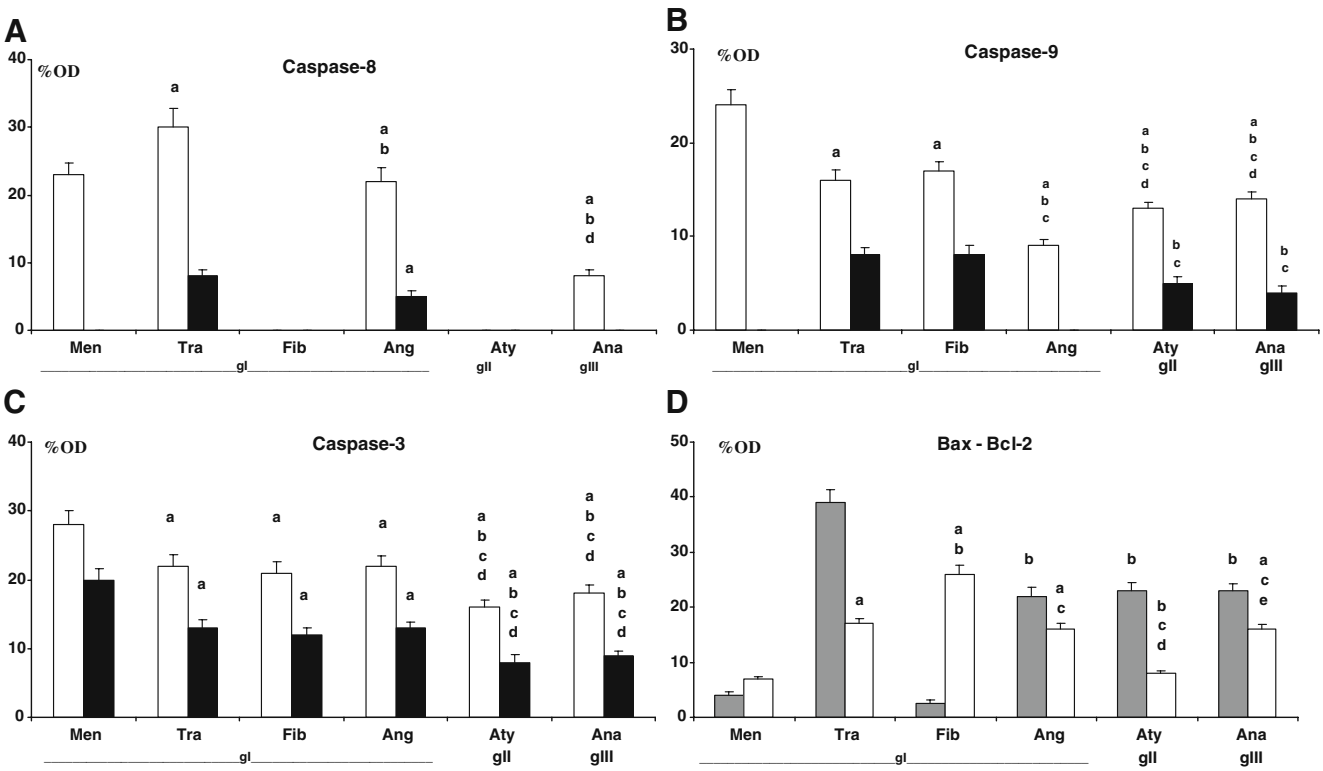

Fig. 5 Bar histogram showing the densitometric analysis of western blot band. **A**, **B**, **C**: *white bar*= pro-caspase expression; *black bar*= active fragment caspase expression. **D**: *white bar*= bcl-2 expression; *grey bar*= bax expression. *a*= $p<0.05$ vs. Men; *b*= $p<0.05$ vs. Tra; *c*= $p<0.05$ vs. Fib; *d*= $p<0.05$ vs. Ang; *e*= $p<0.05$ vs. Aty

meningiomas caspase-6 proform is expressed but not activated. Definitely these findings together the expression of apoptotic inhibitors factors may point out a change in the efficiency of apoptotic machinery of cell death.

Nevertheless apoptotic cell death may occurs even in the presence of broad spectrum of caspase inhibitors [33], supporting our findings about the occurrence of a low number of apoptotic cells (visualized as T+casp3-a.f. positive cells) in grade I meningiomas. Instead in meningotheliomatous meningiomas the high expression of active caspase-3 is associated to the lack of TUNEL positive nuclei or others apoptotic signal activation. The caspase-3 activation has been recognized in a close association also with intracellular events involved in differentiation and proliferation pathways [10, 34]. Otherwise the expression of apoptotic inhibitor XIAP and antiapoptotic bcl-2 factor may indicate the activation of cellular protection mechanisms against death pathways eventually occurring. Furthermore inhibitory action of survivin on caspase-3 activity has been debated [11, 35], and survivin has been associated to poor prognosis and resistance to therapy, in particular when linked to high expression of cell proliferation index [5, 11].

We speculate that in grade I meningiomas under study the high expression of capase-3 activation associated to high level of survivin expression sustains the survival of altered tumor cells [5, 36–38], These findings confirm caspase-3 activation detection in grade I meningioma as a useful tools to evaluate a quickly evolution of tumoral mass and a worst level of clinical prognosis [6], although caspase-3 activation should indicate cell proliferation rather than cell death.

The different expression of c-Flip and XIAP might indicate in the different grade I meningiomas the occurrence of different intracellular pathways of surviving and perhaps a different surviving enhancement.

In grade II and III meningiomas the wider decrease of apoptotic signals (proform or activated caspases and apoptotic cells) confirm the wider programmed cell death pathways alteration that seems to accompany cell transformation in more malignant tumor [3, 4].

In grade II and III meningiomas the increase of TUNEL positive cells negative for caspase-3, could well indicate the occurrence of non-apoptotic or necrotic cell death [22], an indicator of ischemic occurrence that the fast increase in tumor mass growing induce on cells. Indeed necrosis in meningiomas is often associated to malignancy grade or more aggressive behaviour [39]. Otherwise in comparison with grade I meningiomas the high level of proliferating index indicates a high rate of

tumor mass growth, and the low expression of survivin may support the greater increase of cell death. In grade II and above all grade III meningioma the high proliferation rate observed represent the main index characterising these tumor,

In conclusion the expression of apoptotic signals in different meningiomas seems to support intracellular pathways related to survival or selection of cellular elements characterising different meningioma types.

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