Journal of Neuroimmunology (2006) 175 (1-2): 59-68. doi:10.1016/j.jneuroim.2006.03.006

T-cell Apoptosis in Human Glioblastoma Multiforme: Implications for Immunotherapy

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Abstract

We used immunohistochemistry and flow cytometry to assess apoptosis in human glioblastoma multiforme (GBM). Our immunohistochemical study revealed apoptosis of glioma cells expressing glial fibrillary acidic protein and of $CD3^+$ T cells infiltrating GBM. To quantify and phenotype the apoptotic T cells, we performed flow cytometry on lymphocytes separated from GBM. The cells were stained with annexin-V-FLUOS/propidium iodide to identify apoptosis. We found that high proportions of both the $CD4^+$ and $CD8^+$ T cells were apoptotic. In particular, we found that T cells expressing Fas ligand (Fas-L, CD95L) were eight times more vulnerable to apoptosis than those not expressing Fas-L, which suggests that the T-cell apoptosis is induced by overactivation of the T-cell receptor, possibly in the absence of appropriate costimulation. Our results have implications for the design of immunotherapies for GBM.

Keywords: Glioblastoma; T cell; apoptosis; tumour; Fas (CD95)

1. Introduction

Apoptosis is a form of controlled cell death that occurs under numerous physiological and pathological conditions, including in glioblastoma multiforme (GBM) (Kuriyama et al., 2002 and Schiffer et al., 1995). It is generally assumed that the apoptotic cells within GBM are tumour cells themselves. Indeed, the rate of apoptosis has been linked to prognosis by some authors (Kuriyama et al., 2002 and Nakamizo et al., 2002). However, the presence of apoptotic cells within GBM may be more complex than previously assumed since recent evidence has suggested that the T lymphocytes infiltrating GBM may also undergo apoptosis (Didenko et al., 2002).

Glioma patients are known to have a depressed immune system (Dix et al., 1999). The nature of this immunodeficiency is complex and incompletely understood, but apoptosis of tumour-infiltrating lymphocytes may be a significant factor. In this study we used immunohistochemistry to identify apoptosis in both tumour cells and T cells, and performed flow cytometry of separated lymphocytes to quantify and phenotype apoptotic T cells in GBM. Our immunohistochemical studies demonstrated apoptosis of T cells and glioma cells, and flow cytometry revealed a high frequency of apoptosis in both $CD4^+$ and $CD8^+$ T cells infiltrating GBM, particularly in T cells expressing Fas ligand (CD95 ligand; Fas-L). Our results are likely to have implications for the design of vaccine therapies for GBM.

2. Methods

2.1. Tumour specimens

Tumour specimens were handled routinely for histological analysis and graded by a neuropathologist according to the WHO system (Kleihues et al., 1993). Fifteen specimens that were identified as glioblastoma multiforme (WHO grade IV, GBM) were used in this study. Freshly resected samples of these tumours were taken directly from the operating room for processing, that included cell separation and flow cytometry analysis.

2.2. Examination of semithin sections

Three of the 9 tumours used for immunohistochemistry (see below) were also examined using semithin sections. Small fragments of tumour (2–4 mm diameter) were fixed in glutaraldehyde. Subsequently they were embedded in epoxy resin and 1–2 μm sections were cut. Sections were mounted on glass slides, stained with methylene blue and examined at high power with a light microscope.

2.3. TUNEL analysis, immunohistochemistry and dual labelling

Nine tumours were analyzed by immunohistochemistry. Tumour specimens were cut into small blocks and fixed in formalin, then embedded in paraffin. Six micrometer-thick sections were deparaffinized with xylene, rehydrated in graded concentrations of alcohol, and washed in water. Antigen retrieval was performed by microwaving at medium power in 0.01 M sodium citrate (pH 6). Non-specific labelling was inhibited by incubating the slides with a blocking solution (Tris-HCl, 0.1 M, pH 7.5 containing 3% bovine serum albumin (BSA) and 20% normal bovine serum) for 30 min at room temperature.

To analyze tissue sections for apoptosis, the TUNEL method was used. Although TUNEL can label necrotic cells as well as apoptotic cells (Gold et al., 1994), this problem is easily overcome by assessing the distribution of positive TUNEL cells and correlating the slides with sections stained with haematoxylin and eosin (H and E). We used a commercially available TUNEL-staining kit (Roche Applied Sciences) and followed the manufacturer's instructions, including the use of positive and negative controls.

Subsequent to TUNEL staining, sections were immunostained with astrocyte-specific or T-cell-specific antibodies. Labelling using primary antibodies (anti-GFAP and anti-CD3, mouse anti-human, 1:50, BD Biosciences) occurred at 4 °C overnight. After washing the slides several times with phosphate-buffered saline, a secondary antibody was applied (biotinylated goat anti-mouse, 1:500, BD Biosciences) at room temperature for 1 h. The signal was visualized using streptavidin-Texas Red conjugates (1:500, Roche Applied Sciences; 30 min at room temperature). Negative control experiments in which no primary antibody was added, were performed in each batch of staining experiments. Autofluorescence was quenched by treating the slides with Sudan Black B (0.3% in 70% ethanol) for 5 min at room temperature before TUNEL staining and after immunolabelling.

Slides were visualized using alternate filters on a fluorescent microscope, and images were recorded using a digital camera. Corresponding images were subsequently fused using Adobe Photoshop® computer software. Given the potential inaccuracies, we made no attempts to quantify the degree of apoptosis using immunohistochemistry.

2.4. Magnetic separation of T cells and analysis of apoptosis by flow cytometry

Fresh tumour tissue was taken from the operating room in Hartmann's solution. The tumour was immediately cut into small pieces in cell culture medium (RPMI/5% FCS). Cells were then passed through a cotton wool filter to remove debris, and counted using a haemocytometer before magnetic separation and flow cytometry. Enzymatic digestion was not used. Six tumours were analyzed in this way. A further 3 tumours, because of a greater availability of tissue, underwent isotype-control analysis before and after cell separation (see below).

Some of each specimen was kept for analysis of the whole tumour. The remainder was used for magnetic separation of $CD3^+$, $CD4^+$ and $CD8^+$ cells using the manufacturer's instructions (Miltenyi Biotec). In brief, cells were resuspended in 90 μl of buffer (phosphate-buffered saline (PBS) with 0.5% BSA and 2 mM EDTA, pH 7.2) to which 10 μl of the primary antibody (anti-CD3, anti-CD4 or anti-CD8, mouse anti-human, BD Biosciences) was added. The cells were then incubated at 4°C for 10 min, washed with buffer, centrifuged and then resuspended in 80 μl of buffer. Twenty microlitres of magnetic-bead labelled secondary antibody (goat-antimouse IgG microbeads, Miltenyi Biotec) were then added and the cells were then incubated at 4 °C for 15 min. Subsequently, the cells were washed in buffer and passed through a magnetic column according to the manufacturer's instructions. Negative and positive cells were collected.

After separation of $CD3^+$, $CD4^+$ and $CD8^+$ cells, specimens were analyzed for apoptosis using annexin-V-FLUOS/propidium iodide labelling and flow cytometry using a commercially available kit. In brief, cells were centrifuged and labelled with a mixture of annexin-V conjugated with fluorescein, and propidium iodide according to the manufacturer's instructions (Roche Applied Science). Cells were analyzed by flow cytometry, and viable cells (annexin-V negative, propidium iodide negative), cells in early apoptosis (annexin-V positive, propidium iodide negative), and cells in late apoptosis or necrosis (annexin-V positive, propidium iodide positive) were identified and counted. A minimum of 5000 cells were counted for each sample.

2.5. Isotype-control experiments

In order to establish that cells positively selected by magnetic separation were specifically labelled by anti-CD3 antibody and not non-specifically labelled, isotype controls were performed before and after separation in 3 tumours. Single-cell suspensions were divided into 2 aliquots (approximately 1×10^7 cells each) – one was labelled with CD3-APC (Becton Dickinson, mouse IgG_1 anti-human) and the other with an isotype-control antibody linked to APC (Becton Dickinson, mouse IgG_1). Labelling was carried out as described above for magnetic separation. After keeping some specimen aside from each aliquot, the labelled cells were then passed through a magnetic column as described above. Positive cells were collected and, alongside the pre-separation specimen from the same aliquot, were analyzed by flow cytometry.

2.6. Four-colour flow cytometry

Three specimens were used for this analysis. Single-cell suspensions were prepared as described above, and then passed through a Ficoll gradient. Cells were then taken from the interface and washed twice with cold PBS. Approximately 1×10^7 cells were then resuspended in cold PBS-azide. To this were added propidium iodide (5 μl, 10 μg/ml), annexin-V PE (BD Pharmingen) (5 μl), anti-CD3-APC (20 μl, BD-Pharmingen) and anti-Fas-

L-FITC (1 μl, Bender MedSystems). The cells were then incubated on ice in the dark for 60 min, centrifuged and washed with cold PBS-azide. Flow cytometry was then performed. Concurrently, isotype-control antibodies were used to control for non-specific binding. Results were compared to peripheral blood mononuclear cells from control subjects so that lymphocytes could be identified by forward- and side-scatter characteristics.

3. Results

3.1. Semithin sections, immunohistochemistry and TUNEL analysis

When semithin sections were examined with high power, apoptotic bodies showing characteristic nuclear fragmentation could readily be identified (Fig. 1a). However, it was not possible, on morphological criteria alone, to identify the cellular origin of these apoptotic cells.

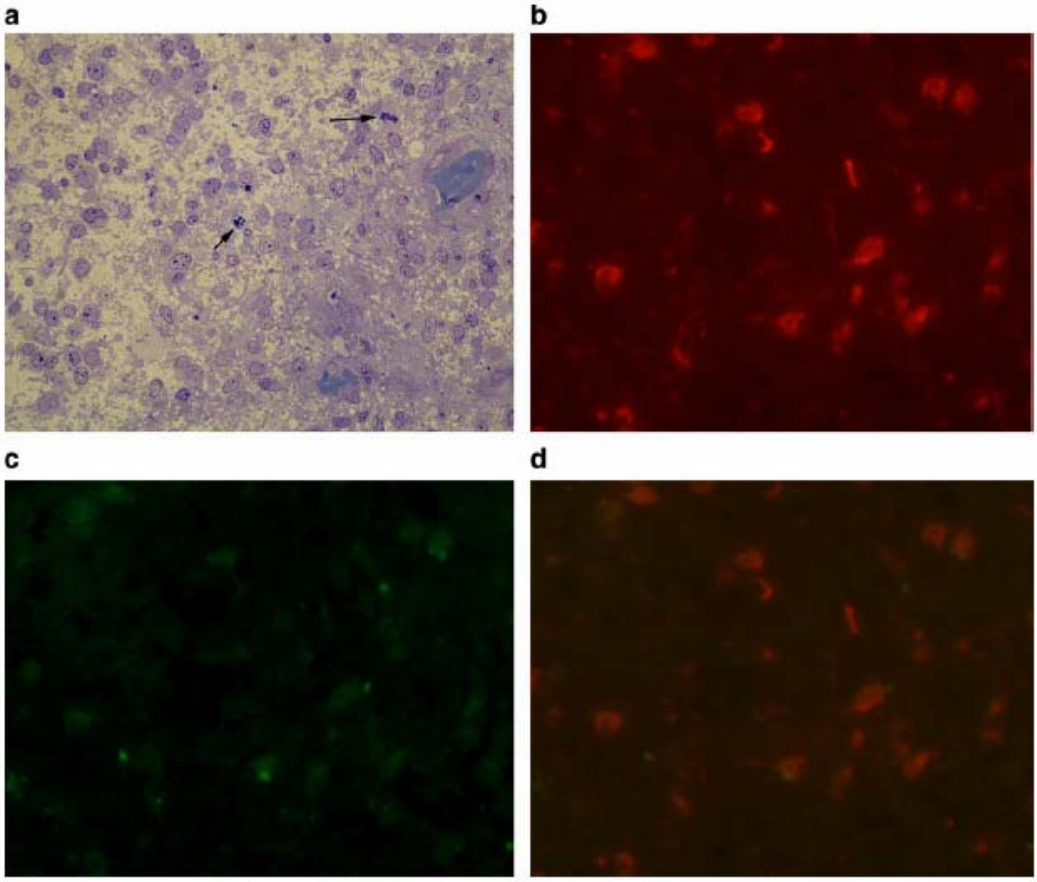

Fig. 1. A GBM is analyzed for the presence of apoptosis using semithin sections and dual labelling immunohistochemistry. a. Semithin section of a GBM illustrating apoptosis with characteristic nuclear fragmentation (short arrow) as well as a mitotic figure (long arrow). The cell of origin of the apoptotic figure is not able to be determined. b–d. Dual labelling immunohistochemistry using anti-GFAP antibody and TUNEL staining. b. Staining with anti-GFAP and visualized with Texas Red. Tumour cells are shown to be GFAP+. c. TUNEL staining of the same slide. Apoptotic bodies are identified by bright green staining. d. When the anti-GFAP and TUNEL images are combined, TUNEL positivity can be seen in $GFAP^+$ tumour cells (red + green = yellow, bottom centre), as well as in $GFAP^-$ cells (green).

Using fluorescent immunohistochemistry, $GFAP^+$ and $CD3^+$ cells could be readily identified within GBM (Fig. 1 and Fig. 2). As is widely recognized, the degree of GFAP positivity was variable, and not all tumour cells were positive using fluorescent immunolabelling. All specimens analyzed showed at least some GFAP reactivity. $CD3^+$ cells were observed in 6 out of the 9 specimens analyzed. These cells were seen scattered throughout the parenchyma of GBM, either as single cells or in small groups, as well as around blood vessels. Overall, $CD3^+$ T cells represented only a few percent of the total number of cells.

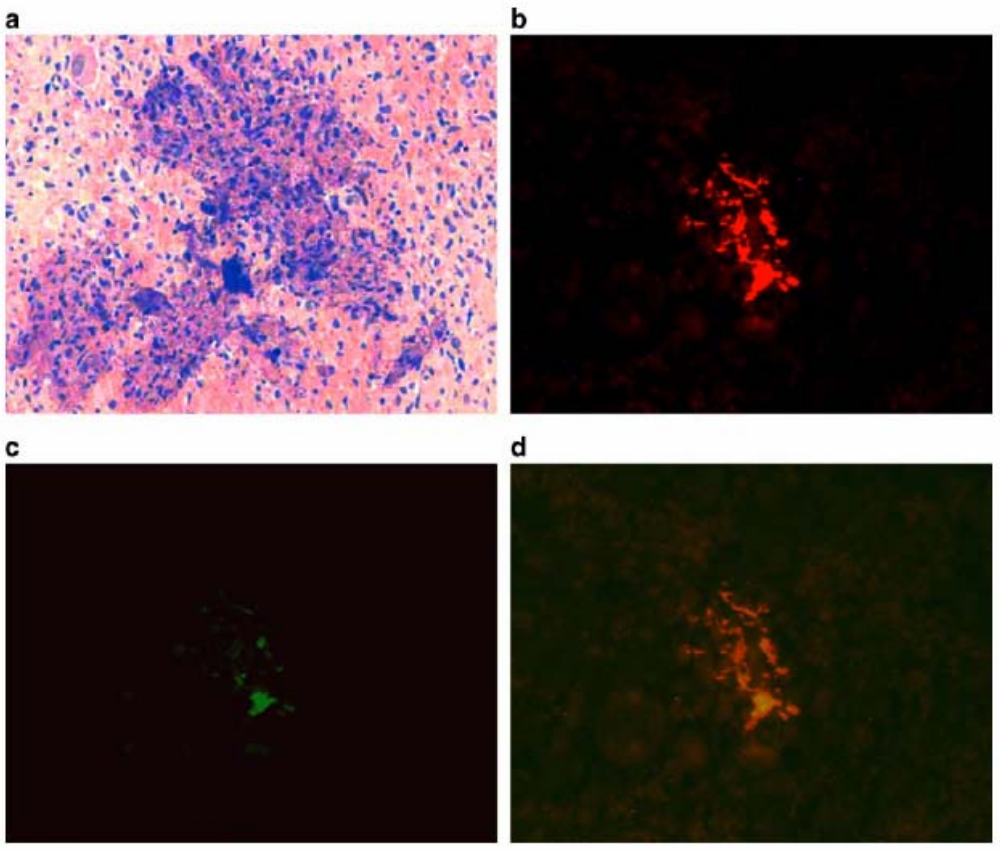

Fig. 2. Dual labelling immunohistochemistry of a GBM using anti-CD3 antibody and TUNEL staining. a. GBM stained with H and E showing a region of endothelial proliferation. b. Immunolabeling with anti-CD3 antibody and Texas Red shows a group of $CD3^+$ cells within this region (red). c. TUNEL staining of the same slide. Several apoptotic cells are seen (bright green). d. When the images are combined, some of the apoptotic cells are shown to be $CD3^+$, i.e. tumour-infiltrating T lymphocytes (yellow).

Using TUNEL analysis, positive cells were seen in all specimens. Areas of necrosis could easily be identified when correlated with H and E slides. Apoptotic cells were identified if the $TUNEL^+$ cell was not within an area of necrosis. In addition, these cells tended to have small, condensed nuclei, a morphological characteristic of apoptosis.

In dual labelling using anti-GFAP as the primary antibody, $TUNEL^+$ cells were seen in $GFAP^+$ (i.e. tumour cells) and $GFAP^-$ cells (Fig. 1). $TUNEL^+/GFAP^-$ cells were seen both in isolation and adjacent to $GFAP^+$ cells. Similarly, when using anti-CD3 as the primary antibody, $TUNEL^+$ cells were seen in $CD3^+$ and $CD3^-$ cells (Fig. 2). Most $TUNEL^+$ cells did not stain for CD3. $TUNEL^+/CD3^+$ cells (i.e. apoptotic T cells) occurred as isolated, intraparenchymal cells as well as in small groups (Fig. 2).

3.2. Single-cell separation, annexin-V/propidium iodide labelling and flow cytometry

Three tumours were analyzed for CD3 staining using APC-labelled anti-CD3 and anti-isotype antibodies, before and after magnetic separation using anti-CD3 as the primary antibody. The results showed that cells positively selected for CD3 expression by magnetic separation did show some increase in non-specific antibody binding. However, using magnetic separation, $CD3^+$ cells could be readily concentrated to a high degree. On average, before magnetic separation, $CD3^+$ cells represented only 1–2% of the cell population; after magnetic separation, $CD3^+$ cells represented almost 70% of the positively selected cell population (Fig. 3). Nonspecific binding could account for only a minority of this.

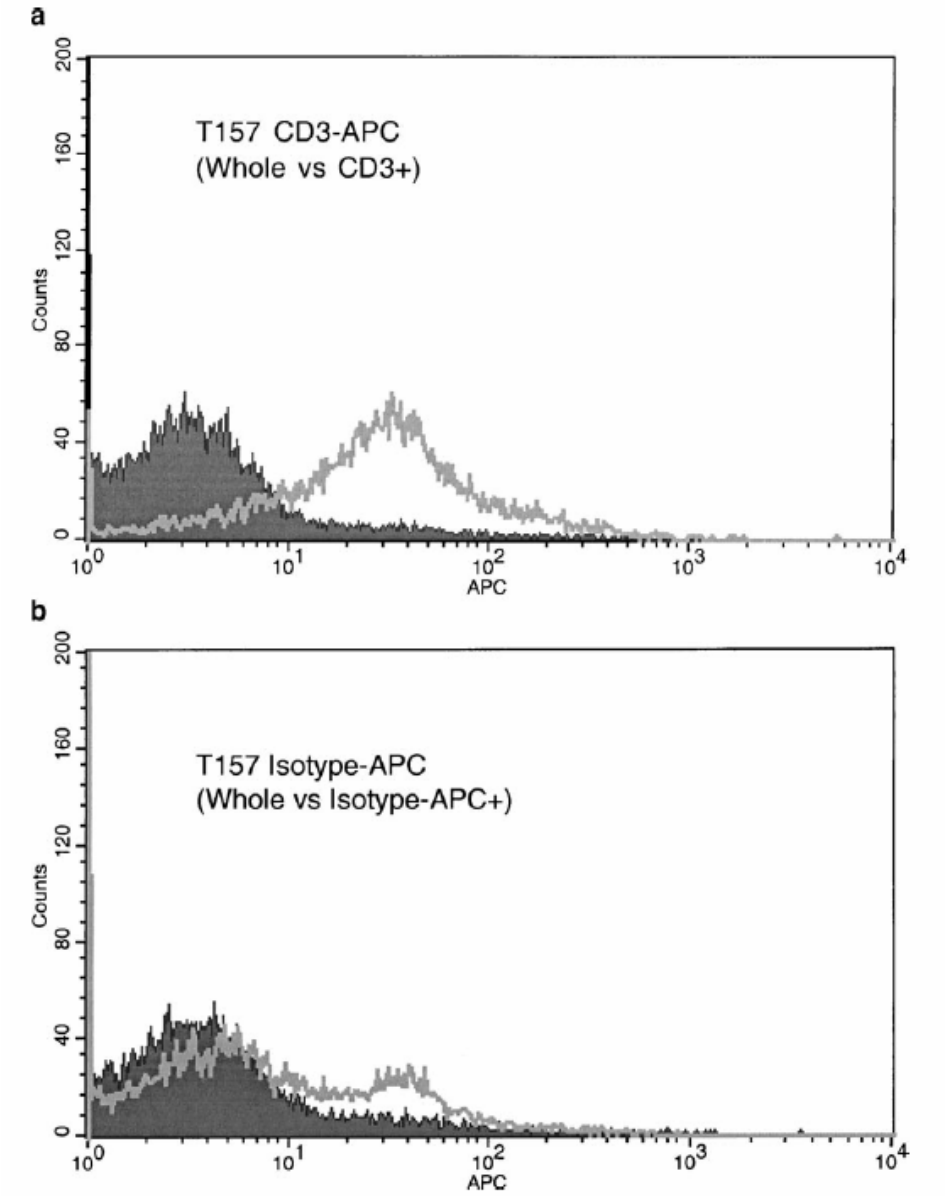

Fig. 3. Flow cytometry detecting APC labelling. A minimum of 10,000 cells were counted in each case. a. Cells were labelled with CD3-APC then passed through a magnetic column. Pre-separation cells (whole) (black) are compared to positive cells (grey). The significant concentration of $CD3^+$ cells by magnetic separation is demonstrated. b. Cells were labelled with isotype-APC and passed through a magnetic column. Positive cells do show a small amount of concentration using this method, consistent with some non-specific labelling.

Six GBM underwent analysis of $CD3^+$ cells, and 3 of these underwent analysis of $CD4^+$ and $CD8^+$ cells. In general, $CD3^+$ cells represented less than 2% of the total number of cells within any given tumour specimen (data not shown). In tumours that were separated according to CD3, CD4 or CD8 expression, the proportions of cells that were viable, were undergoing early apoptosis and those undergoing late apoptosis or necrosis were analyzed. In tumours that were separated according to annexin-V expression, the proportions of $CD3^+$ cells were analyzed in the annexin-V positive and negative fractions.

Using annexin-V/propidium iodide labelling, cells separated on the basis of CD3, CD4 or CD8 positivity showed a consistent decrease in the proportion of viable cells, and an increase in the proportion of cells in early apoptosis and late apoptosis/necrosis (Table 1 and Table 2; Fig. 4 and Fig. 5). For example, when the results of the 6 GBM examined were combined, there was a significant difference in the mean proportions of cells in early apoptosis between $CD3^+$ and $CD3^-$ fractions (22.6% vs. 6.8%, $p < 0.01$). There was also an increased mean proportion of cells in late apoptosis/necrosis in $CD3^+$ fractions, compared to $CD3^-$ cells (33.0% vs 4.6%, $p < 0.01$). Because late apoptosis and necrosis can not be differentiated using this method, we believe the figure for early apoptosis is more significant. Similar results were obtained when $CD4^+$ and $CD8^+$ fractions were compared to $CD4^-$ and $CD8^-$ fractions, respectively (Table 2, Fig. 5). Although the combined results of the 3 tumours examined did not show a statistically significant difference between the means of positive and negative fractions, in each individual example there was a consistent increase in the amount of apoptosis in positive fractions compared to negative cell fractions (CD4: early apoptosis, mean increase = 3.5-fold, SD 0.47; CD8: early apoptosis, mean increase = 3.8-fold, SD 1.3).

Table 1. Summary of results from 6 GBM

Mean (%)	**$CD3^-$**	**$CD3^+$**	***p*-value**
Viable cells	88.2	43.4	< 0.01
Early apoptosis	6.8	22.6	< 0.01
Late apoptosis/necrosis	4.6	33.0	< 0.01

Cells were separated by magnetic labelling and then stained with annexin-V-FLUOS/propidium iodide. Flow cytometry was then performed (see Fig. 3). The results in the table represent the mean percentages from our studies on 6 GBM. A minimum of 5000 cells were counted for each tumour, either as viable (annexin-V negative and propidium iodide negative), undergoing early apoptosis (annexin-V positive and propidium iodide negative) or undergoing late apoptosis or necrosis (annexin-V positive and propidium iodide positive). *P*-values are for Student's *t*-test (unpaired, 2-tailed).

3.3. Analysis of four-colour flow cytometry

Three GBM were analyzed in this fashion. By comparing tumour-infiltrating lymphocytes to peripheral blood mononuclear cells, lymphocytes could be identified on the basis of forward- and side-scatter characteristics. Lymphocytes were then further analyzed. Cells that stained with propidium iodide (i.e. necrosis or late apoptosis) were excluded from further analysis. The remaining cells were examined on the basis of CD3-APC, Fas-L-FITC and Annexin V-PE staining. Of cells that were $CD3^+$, 27% were Annexin-V PE^+ (range 21–35%). Of $CD3^+$ cells, 59% were Fas-L^+ (range 55–65%), and 41% were Fas-L^-. For $CD3^+$ Fas-L^+ cells, 41% were Annexin V-PE^+ (range 36–49%), compared to 5% that were $CD3^+$ Fas-L^- (range 4–7%) (Fig. 6). This indicates that expression of Fas-L increases the susceptibility of T cells to apoptosis by a factor of 8.

4. Discussion

In this study we have analyzed T-lymphocyte apoptosis within GBM by immunohistochemistry and flow cytometry of separated lymphocytes. Our immunohistochemical studies revealed apoptosis of both T cells and glioma cells in GBM. Flow cytometry demonstrated that high proportions of $CD4^+$ T cells and $CD8^+$ T cells were apoptotic and that T cells expressing Fas-L were much more likely to undergo apoptosis than those not expressing Fas-L.

Table 2. Summary of results from 3 GBM that were analyzed for apoptosis after separation of $CD4^+$ cells and $CD8^+$ cells

Mean (%)	**$CD4^-$**	**$CD4^+$**	***p*-value**	**$CD8^-$**	**$CD8^+$**	***p*-value**	***p*-value $CD4^+$ vs $CD8^+$**
Viable	90.1	44.7	0.15	90.8	44.2	0.18	0.98
Early apoptosis	7.12	25.6	0.19	6.4	23.3	0.18	0.87
Late apoptosis	2.8	29.7	0.12	2.8	32.5	0.19	0.88

Figures represent the mean percentages of a minimum of 5000 cells counted. *P*-values for Student's *t*-test (unpaired, 2 tailed) are given.

The presence of apoptosis within high-grade glial tumours has been well recognized (Kuriyama et al., 2002 and Schiffer et al., 1995). Our study using dual-labelling immunohistochemistry has shown that apoptosis does occur in GFAP-positive tumour cells, a fact that, as far as we can determine, has not been previously been shown using this methodology. Two recent papers have found a positive association between apoptotic index and survival (Kuriyama et al., 2002 and Nakamizo et al., 2002). Earlier studies also showed an improved survival with increased apoptosis (Korshunov et al., 1999 and Rhodes, 1998) although others have not found such a relationship (Heesters et al., 1999 and Nakamura et al., 1997). This inconsistency of results may be related to the variable identity of apoptotic cells, which has not been widely recognized. The common assumption has been that apoptotic bodies represent tumour cells, yet within GBM there exists a heterogeneous cell population that includes tumour cells, endothelial cells, leukocytes and normal brain cells. Apoptosis could potentially occur in any of these cell populations.

Significant lymphocytic infiltrates have been previously observed in between 11% and 58% of high-grade gliomas (Bertrand and Mannen, 1960, Palma et al., 1978, Ridley and Cavanagh, 1971 and Schiffer et al., 1974). Some authors also observed that patients with more prominent lymphocytic infiltrates had a better survival (Brooks et al., 1978 and Palma et al., 1978). Using cell separation and flow cytometry, we identified $CD3^+$ T cells in all tumours examined, but by immunohistochemistry observed $CD3^+$ cells in only 6 of 9 GBM, a figure in keeping with previous histological studies (Bertrand and Mannen, 1960 and von Hanwehr et al., 1984). Flow cytometry is likely therefore to be more sensitive and thus has a significant advantage when examining the T-cell phenotype. It is worth noting, however, that since T cells represent only a small fraction of the total cell population within a GBM, the contribution of apoptotic T cells to the total number of apoptotic cells is likely to be small.

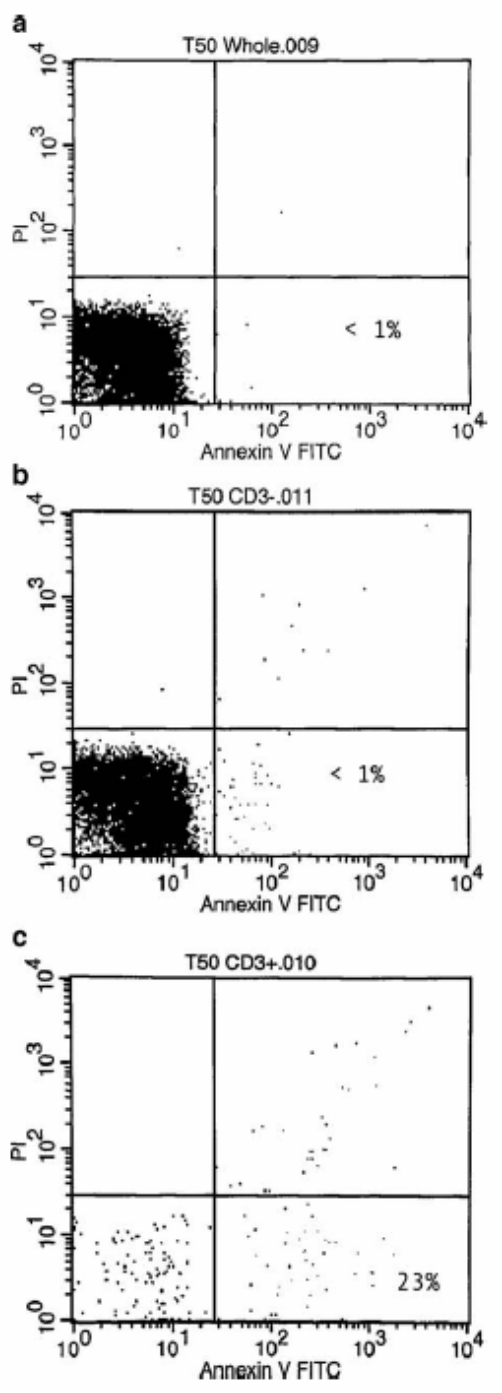

Fig. 4. Flow cytometry after Annexin-V FLUOS/propidium iodide (PI) staining. The bottom left quadrant (both annexin-V and propidium iodide negative) are viable cells. Annexin-V positive and propidium iodide negative cells (bottom right quadrant) are early apoptotic cells. Late apoptosis/necrosis is represented in the top right quadrant (both annexin-V and propidium iodide positive). a. Analysis of a single-cell suspension prepared from a GBM (T50) shows less than 1% of cells in early apoptosis. b. Analysis of the same tumour after magnetic cell separation (see Materials and methods for details). The $CD3^-$ fraction also shows less than 1% of cells in early apoptosis. c. $CD3^+$ cells (i.e. tumour-infiltrating T lymphocytes) in this same tumour showed 23% of cells were in early apoptosis.

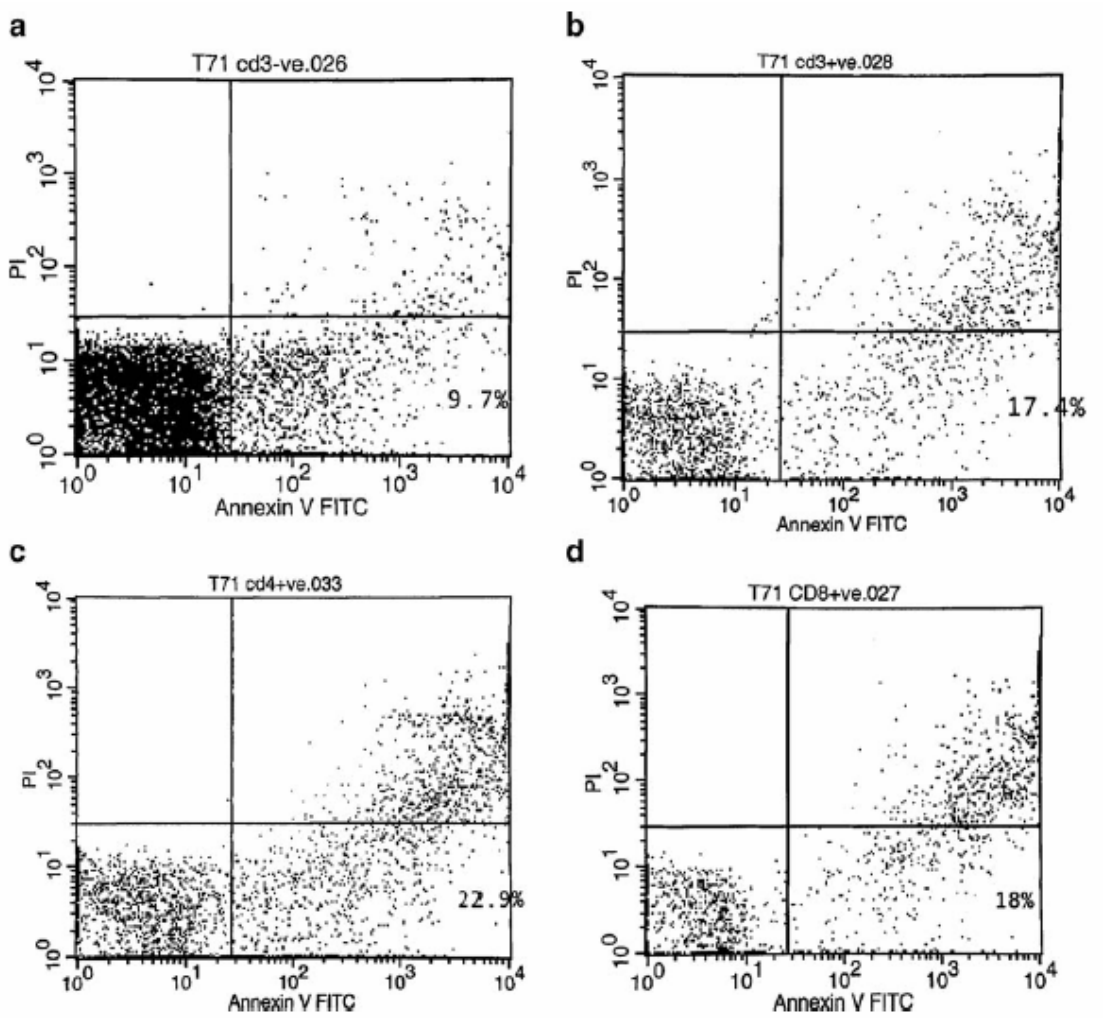

Fig. 5. Flow cytometry after Annexin-V FLUOS/propidium iodide (PI) staining. This GBM (T71) was analyzed after separation of $CD3^+$ cells, $CD4^+$ cells and $CD8^+$ cells. $CD3^-$ cells showed a lower rate of early apoptosis (a, 9.7%) compared to $CD3^+$ cells (b, 17.4%), $CD4^+$ cells (c, 22.9%) and $CD8^+$ cells (d, 18%).

It is clear that effective anti-tumour cell-mediated immune responses are not generated in patients with glioma. It has long been recognized that patients harbouring malignant gliomas exhibit depressed cellular immune responses compared to healthy individuals (Mahaley et al., 1983 and Young et al., 1976). Although gliomas express tumour-associated antigens (Parney et al., 2000), their ability to present these antigens to T cells is controversial. This may relate to their major histocompatibility complex (MHC) expression. Because gliomas are likely to express only low levels of class I MHC molecules (Miyagi et al., 1990 and Saito et al., 1988), $CD8^+$ cytotoxic T cells may be relatively ineffective in eliminating glioma cells. Class II molecules are generally expressed by professional antigen-presenting cells (Ni and O'Neill, 1997). Within the CNS, microglia are the usual class II MHC-positive cells (Gehrmann et al., 1995 and Theele and Streit, 1993). Microglia and macrophages are present in increased numbers within human gliomas in situ (Leung et al., 1997, Roggendorf et al., 1996 and Rossi et al., 1987). However, the absence of antiglioma immunity suggests that these microglia are not functioning as effective immunostimulating cells. Indeed it is possible that they may be subverted by the tumour into secreting factors that support glioma growth (Mantovani et al., 1992). Although transforming growth factor-β (TGF-β) produced by gliomas has been suggested to have a T-cell immunosuppressant role, the concentration of TGF-β in culture supernatants obtained from tumours does not correlate with the concentrations of TGF-β required to inhibit T-cell function in vitro (Dix et al., 1999).

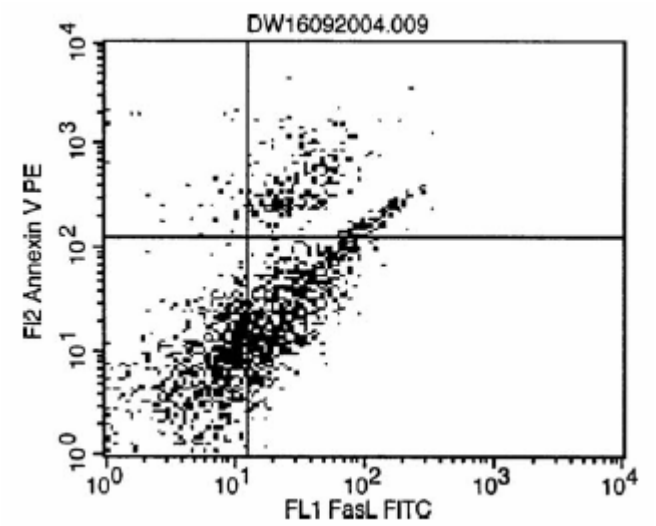

Fig. 6. Four-colour flow cytometry of tumour-infiltrating lymphocytes after excluding propidium iodide positive (PI^+) cells and analyzing only $CD3^+$ cells. The graph represents Annexin V-PE^+ cells vs $FasL^+$ cells. This demonstrates that the proportion of $FasL^+$ T cells that are apoptotic is much greater than the percentage of $FasL^-$ T cells that are apoptotic.

Although gliomas are infiltrated by lymphocytes to varying degrees, it has been reported that glioma-infiltrating lymphocytes are inactivated (Black et al., 1992). Using flow cytometry, we have shown that less than half of the T cells infiltrating GBM are viable, and that an average of 22.6% are in early apoptosis. Didenko et al. (2002) demonstrated apoptosis of T cells infiltrating GBM by immunohistochemistry using caspase-3 as a marker of apoptosis and anti-CD3 for labelling T cells. They estimated that 10% of T cells infiltrating GBM were apoptotic. T-cell subsets were not examined in their study. Our immunohistochemical study using TUNEL staining demonstrated apoptosis of T cells and glioma cells in GBM. Our use of lymphocyte separation and flow cytometry yielded a more accurate estimate of the frequency of T-cell apoptosis. We found that high proportions of both $CD4^+$ T cells and $CD8^+$ T cells are apoptotic. Our flow cytometry data on magnetically separated T cells was supported by our data from four-colour flow cytometry; there was a reasonable concordance between the two methods in estimating the percentage of apoptotic T cells. Apoptosis of tumour-infiltrating lymphocytes has also been demonstrated in other tumours, including melanoma, colon cancer and hepatocellular carcinoma (Hahne et al., 1996, O'Connell et al., 1996 and Strand et al., 1996).

T-cell apoptosis has been identified as a critical process in recovery from immune-mediated CNS damage (Pender et al., 1992, Tabi et al., 1994 and Pender and Rist, 2001). Apoptotic T cells in the CNS are phagocytosed by macrophages, microglia, astrocytes and oligodendrocytes (Nguyen and Pender, 1998 and Magnus et al., 2002). It has been proposed that activation-induced apoptosis of previously activated autoreactive T cells in the target organ is a major mechanism for maintaining tolerance (Pender, 1999). Activation-induced apoptosis (i.e. apoptosis of T cells triggered by activation of the T-cell receptor) is mediated through the Fas (CD95) pathway. Activation of T lymphocytes increases their expression of Fas and induces their expression of Fas-L within 24 h, and they become susceptible to Fas-mediated apoptosis 3–4 days after activation (Brunner et al., 1995, Ju et al., 1995 and Dhein et al., 1995). The ligation of Fas on the surface of the T cell can be mediated by Fas-L on the same T cell or by Fas-L expressed by other cells (Brunner et al., 1995 and Dhein et al., 1995). Using four-colour flow cytometry on lymphocytes extracted from GBM, we have shown that T cells expressing Fas-L are 8 times more likely to undergo apoptosis than those not expressing Fas-L. Although we did not measure Fas expression on apoptotic T cells, Fas is known to be expressed on apoptotic T cells in GBM (Didenko et al., 2002). Our finding that T

cells expressing Fas-L are much more likely to undergo apoptosis than T cells not expressing Fas-L is similar to what occurs in the CNS during spontaneous recovery from experimental autoimmune encephalomyelitis (White et al., 1998) and suggests that T-cell apoptosis in GBM is activation-induced and is occurring via the ligation of Fas by Fas-L on the same T cell. Apoptosis of T cells within GBM may explain, to a significant extent, the ineffectiveness of cell-mediated immunity towards gliomas.

Didenko et al. (2002) demonstrated apoptotic T cells adjacent to Fas-L-expressing glioma cells and concluded that Fas-L expression by glioma cells activates the Fas pathway in infiltrating T cells and leads to T-cell apoptosis as a tumour defence mechanism. It seems unlikely that glioma-cell Fas-L expression is the dominant mechanism leading to T-cell apoptosis because Fas-L expression by glioma cells is by no means universal, either within individual tumours or amongst GBM as a group (Husain et al., 1998 and Saas et al., 1997). An alternative role for glioma cells in inducing T-cell apoptosis is that glioma cells act as non-professional antigen-presenting cells which present tumour antigens to T cells but do not provide a co-stimulatory signal. Co-stimulation by professional antigen-presenting cells inhibits apoptosis in previously activated T cells (Groux et al., 1993 and Liu and Janeway, 1990). Therefore we suggest that apoptotic deletion of T cells in GBM is due to activation-induced apoptosis following antigen presentation by glioma cells and microglia. This mechanism is similar to that which has been suggested to account for apoptosis of autoreactive T cells in the CNS during spontaneous recovery from experimental autoimmune encephalomyelitis, where astrocytes and microglia may act as non-professional antigen-presenting cells (Pender, 1999 and Pender and Rist, 2001). Our hypothesis that T-cell apoptosis in GBM results from overstimulation of the T-cell receptor in the absence of appropriate costimulation is consistent with the findings of Prins et al. (2001) who showed that tumour-infiltrating lymphocytes functioned poorly when tested in vitro, with decreased proliferative activity and other characteristics of defective T cells. These phenotypic changes mirrored changes seen in normal $CD8^+$ T cells cultured with polyclonal mitogens for an extended period. Prins et al. (2001) concluded that T cells were undergoing apoptosis due to over-activation related to the glioma microenvironment and that the T cells were thus not contributing to anti-tumour immunity. Our findings are also consistent with those of Chahlavi et al. (2005) who, using an in vitro glioblastoma model, found that activated T cells are more sensitive to apoptosis than resting T cells and also provided evidence that Fas-L expression by glioma cells is not a major mechanism for T-cell apoptosis.

In conclusion, we have demonstrated apoptosis in high proportions of the $CD4^+$ and $CD8^+$ T lymphocytes infiltrating GBM. Our finding that T cells expressing Fas-L are much more likely to undergo apoptosis than those not expressing Fas-L indicates that the T-cell apoptosis is activation-induced. We suggest that T-cell apoptosis in GBM results from overstimulation of the T cell receptor in the absence of appropriate co-stimulation. The results have implications for the immunotherapy of GBM because they suggest that, without appropriate co-stimulation in the CNS, T cells are unlikely to mount an effective anti-GBM attack.

Acknowledgements

This research has been supported by the Royal Brisbane and Women's Hospital Research Foundation and a Project Grant from the National Health and Medical Research Council of Australia.

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