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Detection of DNA strand breaks associated with apoptosis in human brain tumors

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Abstract Apoptosis occurs spontaneously in a wide variety of neoplasms. However, it is difficult to detect apoptotic cells in routine histological sections because the cells undergoing apoptosis die singly and are then rapidly phagocytosed. Since DNA fragmentation is an important hallmark of apoptosis, visualization of DNA strand breaks in tissue sections provides the means for readily identifying apoptotic cells in situ. We have applied an in situ DNA strand break staining procedure for the quantitative estimation of apoptotic cells in surgical specimens of 62 different brain tumours. Positively stained apoptotic cells were observed in 25 (40.3%) cases and their percentage (apoptotic index) ranged from 0.1 to 8.9. Both fragmented and condensed nuclei of apoptotic cells and apoptotic bodies were stained. In addition, we assessed the proliferative activity of each specimen by immunostaining with the MIB-1 antibody (MIB-1 index) which detects the cell cycle phase-dependent Ki-67 antigen. Brain tumours with higher MIB-1 indices showed a tendency to higher apoptotic indices. The results of this study indicate that apoptosis occurs spontaneously in various brain tumours.

Key words Brain tumor · Apoptosis · DNA strand breaks · MIB-1 antibody

Introduction

Apoptosis is commonly observed under a wide range of physiological conditions [32]. It is described as a morphological pattern of cell death, characterized by cell shrinkage, membrane blebbing, chromatin condensation and presence of apoptotic bodies. The morphological changes in nuclei that accompany apoptosis are associated with the activation of a specific endogenous endonuclease that breaks the double-strand DNA of chromatin at the internucleosomal linker DNA [12, 31]. On gel electrophoresis these DNA cleavage products display a ladder pattern of multiples of 180–200 base pairs (bp). This type of pattern is considered to represent a useful biochemical indicator of apoptosis [2, 18]. Although apoptosis also occurs spontaneously in certain solid tumours [12, 15, 27], ionizing radiation [5], heat-shock [9] and a variety of unrelated cancer chemotherapeutic agents [10, 14, 26] can trigger apoptosis of tumour cells. In the final stage of apoptosis, dying cells are segmented into apoptotic bodies and phagocytosed by neighbouring cells and macrophages, with no overt inflammatory reaction. Consequently, the induction of apoptosis appears to be a potentially useful modality for the treatment of tumours in general and brain tumours in particular.

The in situ identification of individual cells undergoing apoptosis is rather difficult [13] and several approaches have been suggested for their detection in tissue specimens. Thus, Gorczyca et al. [8] and Gavrieli et al. [6] recently devised an in situ DNA strand break staining method (TUNEL) for detecting apoptotic cells in tissue sections. The method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3′-hydroxyl termini of DNA and the TdT-catalysed repetitive addition of several thousand normal or modified mononucleotides to these 3′-termini.

In this report we present a slight modification of the TUNEL staining method and its application to the in situ detection of DNA strand breaks in brain tumours. The data obtained are expressed as an "apoptotic index" (AI) that represents the percentage of positively stained nuclei in a given specimen. The AI of each sample was compared to the specimen's proliferating activity by immunostaining with the MIB-1 antibody. This antibody reacts with Ki-67, a nuclear antigen that is differentially expressed by cells in the G_1, S, G_2 and M phases of the cell cycle [7, 25].

Materials and methods

This study was carried out on surgical specimens of 62 tumours originating in the central nervous system. The tissue samples were fixed in 3.5% buffered formaldehyde and embedded in paraffin.

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Sections 6 μm thick were deposited on poly-L-lysine-coated slides (MUTO Pure Chemical Co., Tokyo, Japan), deparaffinized, rehydrated to water, and washed with distilled water (DW). The nuclear proteins were removed by incubating the sections for 20 min at room temperature (RT) with 20 μg/ml proteinase K (Sigma, St. Louis, Mo., USA) followed by washing the slides in DW. Endogenous peroxidase was quenched by covering the sections with 2% H_2O_2 for 5 min at RT. The slides were then rinsed with DW, immersed in TdT buffer (0.5M sodium cacodylate, 10 mM $CoCl_2$ and 1 mM dithiothreitol), covered with TdT (0.3 U/ml; Gibco BRL, Gaithersburg, Md., USA) and biotinylated dUTP (0.4 nmol/ml; Boehringer Mannheim, Mannheim, Germany) in TdT buffer and incubated in a humid atmosphere for 60 min at 37° C. The reaction was termined by placing the slides in TB buffer (30 mM sodium citrate, 300 mM sodium chloride) for 30 min at RT. They were then washed with DW and covered for 10 min at RT with 10% normal rabbit serum to block non-specific antibody binding. After washing in DW and immersing in phosphate-buffered saline (PBS), the sections were incubated with peroxidase-conjugated streptavidin (Nichirei, Toyko, Japan) for 30 min at RT and then stained with a mixture of 0.2 mg/ml 3,3′ diaminobenzidine tetrachloride and 0.005% H_2O_2 in 50 mM TRIS-HCl buffer (DAB/H_2O_2). The slides were counterstained with haematoxylin, dehydrated and mounted.

The tissue sections pre-treated for 10 min with 0.7 mg/ml DNase I (Amresco, Solon, Ohio, USA) in sodium cacodylate buffer, pH 7.2, served as positive controls. After extensive washing in DW, these slides were processed according to the protocol mentioned above. Because apoptosis is frequently observed in rat intestine, we also employed the tissue sections from this source as positive controls. Tumour samples subjected to all reaction steps, but incubated with buffer from which TdT was omitted were used as negative controls. The percentage of positively stained nuclei to total nuclei (AI) was determined by counting a total of at least 1000 nuclei in randomly chosen field under high power of light microscopy (×400). The areas with obvious necrosis were excluded.

Tumour sections 6 μm thick were cut, mounted on poly-L-lysine-coated slides, deparaffinized and then rehydrated to water. The slides were washed in DW and placed in a beaker filled with 10 mM citrate buffer (pH 6.0). The beaker was covered with a polyethylene sheet and heated in a household microwave oven (810 W) for 12 min, followed by rinsing the slides in DW and immersion in PBS. After blocking endogenous peroxidase by a 5 min immersion in 2% H_2O_2, the slides were washed three times (5 min each) with PBS and then covered for 10 min at RT with 10% normal rabbit serum (in PBS) to block non-specific antibody binding. The slides thus treated were incubated for 60 min with a 1:40 dilution of the MIB-1 monoclonal antibody (Medical and Biological Laboratories, Nagoya, Japan), washed three times in PBS and exposed to biotinylated rabbit anti-mouse IgG (Nichirei) for 30 min at RT. After washing in PBS, the slides were incubated for 30 min with peroxidase-conjugated streptavidin followed by visualization of antibody binding with the DAB/H_2O_2 solution. The slides were counterstained with haematoxylin, dehydrated and mounted. The percentage of positively stained nuclei (MIB-1 index) was determined as indicated above for the in situ DNA strand break staining procedure.

Statistical evaluation of quantitative data from two groups was carried out using Student's *t*-test.

Results

The DNA strand break staining method is considered to be useful in identification of apoptotic cells; most cells in brain tumours pre-treated with DNase I revealed positive nuclear staining (data not shown). In addition, intense nuclear positivity was also seen in the cells at the tips of the villi in the small intestine of rats. In contrast, no definite staining was seen in specimens exposed to all steps of the procedure, except incubation with TdT. No positive reaction was noted in neuronal, glial and vascular cells in normal brain tissues (data not shown).

The results obtained from the 62 brain tumours examined are summarized in Table 1. Fifteen glioblastomas and 8 astrocytomas showed a randomly scattered distribution of positively stained nuclei. Intense staining was observed in condensed nuclei and fragmented chromatin (Fig. 1A). The apoptotic index was higher in glioblastomas than in astrocytomas. Although the stained nuclei were often observed in pseudopalisading areas, there was no or only very faint staining in the necrotic regions of glioblastomas. Furthermore, no staining was detected in lymphocyte and mitotic cell nuclei with condensed chromatin. In medulloblastomas, the stained nuclei were scattered diffusely throughout the sections and most of them displayed specific apoptotic features such as condensation and fragmentation of chromatin. Moreover, the apoptotic bodies phagocytosed by neighbouring cells showed distinct staining (Fig. 1B, arrow). Occasionally, the cells with positive staining of cytoplasm but not of condensed chromatin were observed (Fig. 1B, arrow *head*). Wijsman et al. [28] suggested that cytoplasmic

Table 1 Apoptotic and MIB-1 indices in various brain tumours.

Histology	Number of cases	Apoptotic index			MIB-1 index	
		No. of positive cases	min~max	m̄	min~max	m̄
Glioblastoma	15	8	0.5~4.0	1.1	6.3~40.4	23.5
Astrocytoma	8	2	1.3~1.7	0.4	1.5~33.5	8.5
Meningioma	10	4	0.3~1.0	0.2	0.7~31.7	9.8
Pituitary adenoma	10	3	0.1~4.9	0.9	0.2~ 3.4	1.2
Schwannoma	2	1	0.6	0.3	0.9~ 2.6	1.8
Medulloblastoma	5	4	0.9~5.9	2.4	3.9~42.9	17.1
Malignant lymphoma	3	3	7.7~8.9	8.2	31.2~88.2	64.1
Craniopharyngioma	3	0	/	0	1.0~ 6.6	3.1
DNT[a]	2	0	/	0	4.0~ 6.3	5.2
Central neurocytoma	4	0	/	0	2.0~ 4.0	2.8
Total	62	25				

[a] Dysembryoplastic neuroepithelial tumour

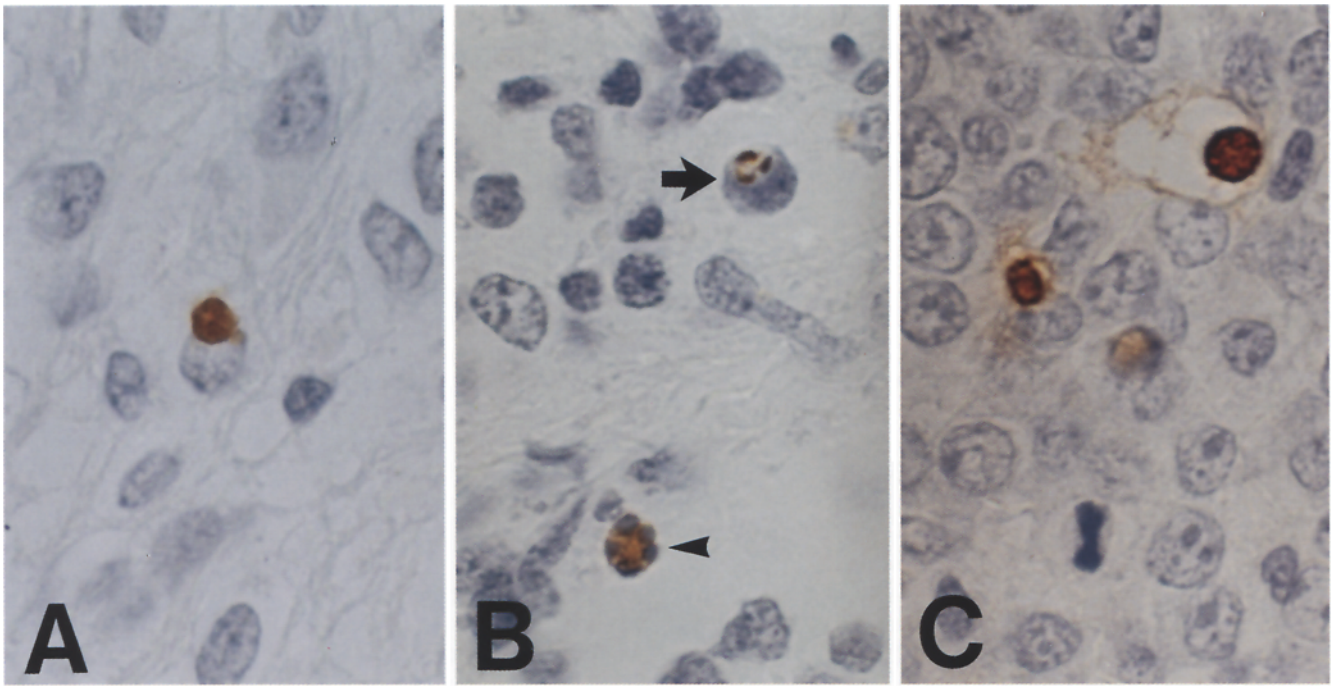
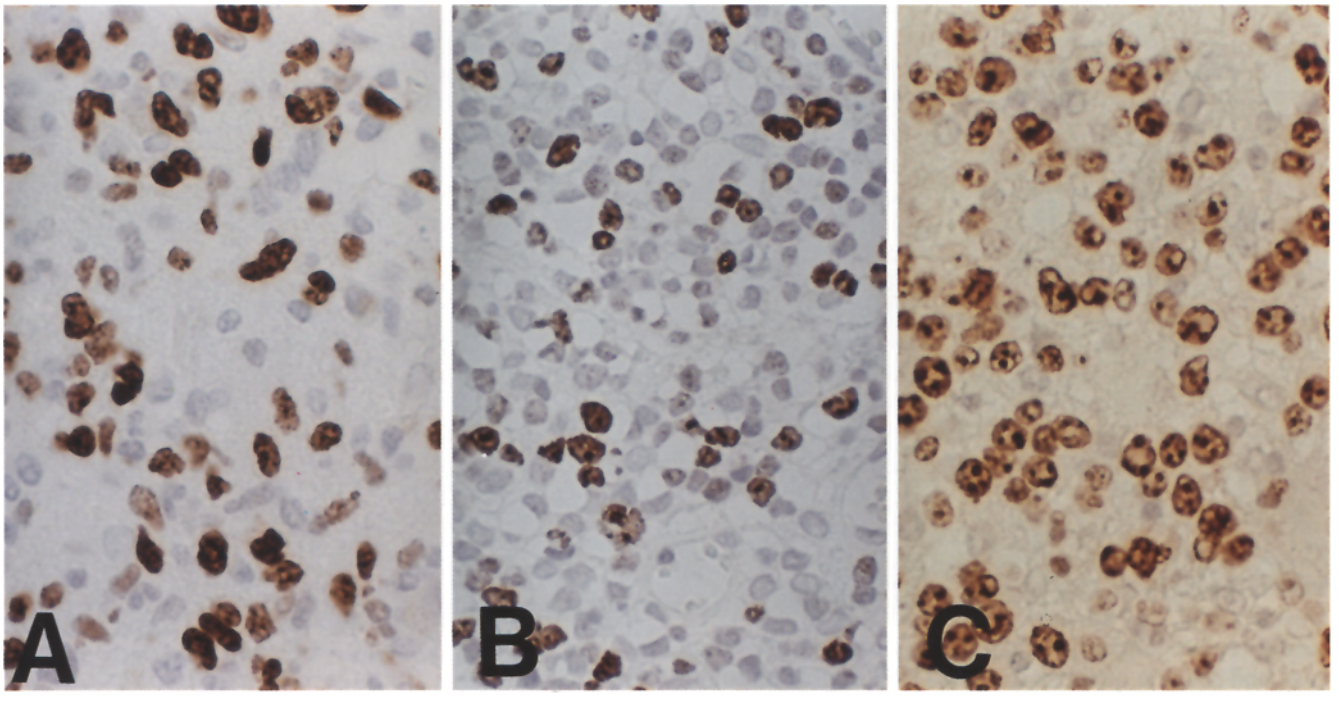


Fig. 1A–C In situ DNA strand breaks staining of brain tumours counterstained with haematoxylin (×1000). **A** Glioblastoma showing intense staining in condensed nuclei. **B** Medulloblastoma. Apoptotic body phagocytosed by a neighboring cell is stained clearly (*arrow*). We excluded the cells stained in only cytoplasm but not in nuclear segments (*arrowhead*). **C** Malignant lymphoma demonstrating intense staining in condensed nuclei and/or chromatin

Fig. 2A–C MIB-1 immunostaining of glioblastoma (**A**), medulloblastoma (**B**) and malignant lymphoma (**C**). The cells positive for MIB-1 immunostaining are clearly distinguishable from the background of unstained cells and show the heterogeneity in nuclear staining of individual cells (×400)

staining is due to leakage of DNA fragments out of the nucleus. This cytoplasmic staining was observed mainly in medulloblastoma. However, we did not take these stained cells into account, because it was very difficult to distinguish them from polymorphonuclear leucocytes. In malignant lymphomas (Fig. 1C), intense staining was observed not only in condensed nuclei and/or chromatin but also nuclear periphery. The AI of benign tumours such as pituitary adenomas and meningiomas was relatively low. No nuclear staining was seen with craniopharygiomas, dysembryoplastic neuroepithelial tumours (DNT) and central neurocytomas. Immunostain-



ing with the MIB-1 antibody revealed that the stained nuclei were clearly distinguishable from the background of unstained cells and that the staining pattern of individual nuclei was heterogeneous (Fig. 2). A significant difference ($P<0.01$) in AI between tumours with less than 10% MIB-1 index (AI: mean±SE, 0.5±0.2) and tumours with more than 10% of MIB-1 index (AI: 2.2±0.6) was found.

Discussion

Apoptosis has recently become a focus of interest in oncology [3, 29]. Apoptosis of tumour cells is known to be triggered by ionizing radiation [5], hyperthermia [9], and a variety of anti-tumour agents [10, 14, 26] and following withdrawal of growth factors [4]. There is evidence that the efficacy of anti-tumour treatment may be associated with the intrinsic ability of tumour cells to undergo apoptosis [3, 29]. Although it has been shown repeatedly that apoptosis also occurs spontaneously in various types of solid tumours [12, 15, 22, 23, 27], its occurrence in brain tumour has not been previously investigated. The successful induction of apoptosis could lead to the development of new anti-tumour strategies and our attempt to detect apoptotic cells in brain tumours represents an initial step along this line.

There are several methods available for the detection of apoptosis [20, 22, 23], which was initially recognized by certain morphological changes in cells. However, since cells undergoing apoptosis die singly and are rapidly phagocytosed, they are not always readily apparent in histological sections. Nevertheless, from the examination of thin resin-embedded tissue sections it had been estimated that the ratio of apoptotic cells to normal cells in experimental tumours ranged between 0.2% and 7.9% [20, 22, 23]. It should be kept in mind that apoptotic cells were sometimes indistinguishable from lymphocytes and mitotic cells by light microscopy. Several biochemical markers of apoptosis have been described [16, 24]. Arends and Wyllie [2] showed that the morphological changes in nuclei associated with apoptosis were due to activation of a Ca^{2+}- and Mg^{2+}-dependent endonuclease that caused double strand breaks at the internucleosomal linker DNA, generating oligonucleotides of approximately 200 bp. At present, the detection of DNA fragmentation by gel electrophoresis is considered to be a satisfactory method by which to obtain information on the chemical features of apoptosis [18]. This is clearly not applicable to identification of individual apoptotic cells in tissue sections; homogenization and DNA extraction are at the cost of tissue structure.

This inherent problem led to the development of the recently described procedures for the in situ detection of extensive DNA strand breaks. Gorczyca et al. [8] and Gavrieli et al. [6] devised a TdT-mediated in situ DNA strand break staining procedure. This method allows an easy and reliable quantification of DNA fragmentation in cells in suspension and in tissue sections. As shown in the present study, both fragmented and condensed nuclei and apoptotic bodies were readily stained, which made it easy to distinguish apoptotic cells from infiltrating lymphocytes or mitotic cells. The AI were relatively high in medulloblastomas with potential capacity of differentiation along neuronal direction [11]. This might be related to the occurrence of massive apoptosis in the developing granule cells of external granular layer [30]. It is obvious that fragmentation of the nuclear DNA also occurs in necrosis, [33] yet we found that necrotic regions in glioblastomas were not or were very faintly stained. These observations are compatible with the notion that DNA strand breaks are less frequent in necrotic cells than in apoptotic cells, since DNA cleavage in necrosis is at random. Consequently, apoptotic cells can be readily distinguished from necrosis [28] by in situ DNA strand break staining. There is a recent publication showing that DNA degradation during apoptosis not always results in internucleosomal fragmentation. In certain cell lines, cleavage of DNA to 50 kbp fragments precedes the appearances of oligonucleosomal fragments during apoptosis [21]. It might be difficult to detect apoptotic cells with only high molecular weight fragments of DNA by this method, because of low incorporation of nucleotides.

Generally, tumour growth takes place by increasing the rate of cell proliferation and/or decreasing rate of cell death [29]. However, growth of certain tumours is closely associated with the loss of the capacity to undergo spontaneous apoptosis, rather than with increased cell proliferation [1, 12]. To ascertain whether this also applies to brain tumours, we immunostained brain tumour sections with the MIB-1 antibody which detected the nuclear Ki-67 antigen differentially expressed by cells in the G_1, S, G_2 and M phases of cell cycle [7]. As shown previously, the percentage of MIB-1 immunostained cells, representing the growth fraction, is considered to be a reliable index for assessing the proliferative activity of a given tumour [25]. As shown here, brain tumours with high proliferative activity (high MIB-1 index) showed higher AI than those with low proliferative activity. In this context Leoncini et al. [17] reported that there was a significant positive correlation between the number of apoptotic bodies and proliferative activity, but the reason for such correlation is unclear. One possible explanation is that accelerated proliferation may cause more frequent DNA duplication and mitotic failures, and that cells with damaged DNA die through an apoptotic process.

We conclude that apoptosis occurs spontaneously in various brain tumours, suggesting the possibility that successful induction of apoptosis might be a potential treatment for cerebral tumours.

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