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Hirotaka Koizumi · Munechika Wakisaka
Koonosuke Nakada · Toshifumi Takakuwa
Teruhiko Fujioka · Noboru Yamate
Toshiyuki Uchikoshi

Demonstration of apoptosis in neuroblastoma and its relationship to tumour regression

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Abstract The in vivo occurrence of apoptosis in neuroblastomas was investigated. Histologically, a number of tumour cells showed typical apoptotic changes, including cell shrinkage, condensed and fragmented nuclei, eosinophilic cytoplasm, and absence of the inflammatory response. These cells coincided closely with the so-called karyorrhectic cells. An electrophoretic DNA ladder, a functional hallmark of apoptosis, was demonstrated in four of six tumours, and DNA fragmentation was detected in situ by terminal deoxytransferase-mediated nick end-labelling in 26 of 35 tumour specimens (74%). The labelled cell counts ranged from 5 to 62 per 5000 tumour cells (mean± SD: 15.0± 14.5). Immunoperoxidase staining revealed that an apoptosis-suppressing protein, *bcl*-2, was expressed abundantly in advanced-stage tumours, whereas it was absent from karyorrhectic-apoptotic cells. Several tumours with the potential for spontaneous regression were *bcl*-2-deficient. Immunostaining of the Fas receptor for apoptosis demonstrated that the tumour cells expressed this molecule on their cell surfaces. Our results provide evidence of apoptosis in neuroblastomas and suggest that *bcl*–2 and the Fas receptor may play a role in its regulatory mechanisms.

Key words Neuroblastoma · Apoptosis · *bcl*-2 · Fas antigen

Introduction

Neuroblastoma (NB) is one of the most common malignant neoplasms of childhood [40]. Generally, the age of the patient and the extent of disease correlate well with survival [7, 11, 35]. However, mortality is spectacularly reduced in some patients, in whom the tumour regresses spontaneously. Stage IV-S ("S" denoting special) has been established for the group of patients who have metastases confined to the liver, skin and/or bone marrow but have a good prognosis with the likelihood of spontaneous regression, usually occurring without treatment [10, 11]. Residual post-surgical disease in children with stage II tumours generally also regresses completely, and the cure rate is over 95% [25]. Furthermore, most patients detected by mass screening for urinary vanillylmandelic acid (VMA) [33] are considered to be in a low-risk subgroup with the potential for spontaneous regression [23]. Consequently, NB has the highest incidence of spontaneous regression among human malignant neoplasms [15, 34]. However, the mechanisms regulating this regression remain to be elucidated [5, 28].

Apoptosis was described initially, by Kerr et al. [20], as a unique type of cell death that plays an important kinetic role in tissue turnover. Apoptosis is considered to be genetically programmed under inhibitory control by the *bcl*–2 oncoprotein [14] and is triggered by a signal(s) via specific receptor(s), such as APO-1 or the Fas antigen [16, 37]. Ultimately the apoptotic signal activates endonuclease(s) causing DNA self-cleavage [39], resulting, morphologically, in cell shrinkage and hyperchromatic nuclear fragments [20, 33] and, biochemically, in chromatin cleavage into nucleosomal oligomers (DNA ladder) [2, 9, 39]. Among the various roles for apoptosis in tumour biology [21], our particular interest is in the fact that apoptosis seems to be capable of leading to tumour regression, which has been suggested to occur with rhabdomyoma of the heart [36], Merkel cell tumour of the skin [19], and infantile myofibromatosis [12]. This raises the strong possibility that spontaneous regression of NB may also be mediated by apoptosis. A similar hypothesis has recently been proposed by Pritchard and Hickman, who suggest that spontaneous regression of NB may be attributed to a delayed turn-on of the developmental time switch for apoptosis in the tumour cells [28]. Studies on apoptosis in NB have been carried out

H. Koizumi (✉) · T. Takakuwa · T. Uchikoshi
The Second Department of Pathology,
St. Marianna University School of Medicine,
Miyamae-ku, Kawasaki 216, Japan

M. Wakisaka · K. Nakada · T. Fujioka · N. Yamate
The Third Department of Surgery,
St. Marianna University School of Medicine,
Miyamae-ku, Kawasaki 216, Japan

only in vitro [17, 24, 27], and no published reports have examined the occurrence of apoptosis in primary NB tissues.

The objectives of the current study were: to ascertain whether apoptosis does occur in NB tissues using histological and biochemical analyses; to investigate expression of the apoptosis-regulating proteins, *bcl*-2 and Fas antigen, in NB; and to assess whether apoptosis participates in NB regression.

Materials and methods

Patients

Tumour specimens were obtained from patients referred to the St. Marianna University Hospital. In total, 35 patient samples, including 7 detected by mass screening [32] were analysed. The tumour specimens were staged according to the Evans staging system [11]: stage I lesions were confined to the organ or structure of origin; stage II lesions extended beyond the organ or structure of origin, but did not cross the midline; stage III extended across the midline; stage IV had remote disease involving the skeleton, organs, soft tissues and/or distant lymph node groups. Stage IV-S tumours were localized primary tumours, as defined for stages I and II, with dissemination limited to the liver, skin and/or bone marrow. Of the 35 patients, 18 (51%) had favourable disease stages (I, II, and IV-S) and 17 (49%) had poor-prognosis disease stages (III and IV; Table 1). Tumour specimens were obtained before the patients underwent chemotherapy. The histopathology of each tumour was classified using the Shimada system, which includes assessment of the patient's age as well as the histological features of the specimens [35].

Histopathology and immunohistochemistry

Formalin-fixed tissues embedded in paraffin were sectioned and stained with standard Harris haematoxylin and eosin (HE). For electron microscopy, the tumours were prefixed in 2.5% (v/v) glutaraldehyde and postfixed in 2% (w/v) osmium tetroxide, and ultrathin sections were cut and stained with uranyl acetate and lead citrate.

For detection of the *bcl*-2 protein, paraffin sections from 35 tumour specimens were immunostained by the streptavidin-biotin peroxidase complex method (LSAB kit, Dako, Copenhagen, Denmark) using a specific monoclonal antibody against the human *bcl*-2 gene product (IgG1, 1 μg/ml; Dako) as a primary antibody. To determine the percentage of *bcl*-2-positive cells in each tumour specimen, the stained cells were counted in five random high-power fields and expressed in relation to the total number of cells counted. The Fas antigen was detected using acetone-fixed cryostat sections from five tumour specimens with a monoclonal antibody against the human Fas antigen (IgM, 1 μg/ml; MBL, Nagoya, Japan) and the LSAB kit. In the negative control preparations, the *bcl*-2 and Fas antibodies were replaced by 1 μg/ml normal mouse IgG1 and IgM respectively.

DNA fragmentation analyses

Frozen materials were obtained from six NBs and a human liver taken from an autopsy case as a control. The DNA was prepared by a proteinase K-sodium dodecyl sulfate method described elsewhere [31] and then separated by electrophoresis through a 1% (w/v) agarose gel. A 1-kb ladder (Gibco, Gainthersburg, Md.) was used as a molecular weight marker.

The in situ nick end-labelling (ISNEL) using the formalin-fixed, paraffin embedded specimens was carried out as described previously [13] with some modifications. Since the extent of fixation varied from specimen to specimen, the protease digestion time was optimized first in each sample. Deparaffinized sections were incubated with 20 μg/ml proteinase K at room temperature (RT) for 5, 10, 15, 30, or 60 min, followed by peroxidase quenching with 3% (v/v) H_2O_2 for 5 min. The sections were then pretreated with DN buffer [13] for 5 min and covered with 200 ng/ml DNase I (Sigma Chemical Co., St. Louis, Mo.) dissolved in DN buffer to introduce DNA breaks in all nuclei. After a 10-min incubation at RT, the slides were washed extensively with double-distilled water, and continued to be processed though ISNEL (described below). Protease treatment was considered to be inadequate when nuclei in the deeper parts of DNAse-treated control sections remained unstained, whereas too strong digestion was characterized by poor morphology and faint staining of non-apoptotic nuclei. Accordingly, most of the preparations were found to show an intensive staining of all nuclei after 10–15 min incubation with proteinase K, although some required a longer digestion time. For ISNEL analysis, adjacent sections were digested for an appropriate time and biotinylated dUTP was tailed to DNA breaks using a terminal deoxytransferase (TdT)-mediated DNA 3′-end labelling kit (Boehringer Mannheim, Mannheim, Germany). The sections were then incubated with streptavidin-peroxidase conjugate (Dako) for 15 min at RT and stained with 0.1% (w/v) 3′, 3′-diaminobenzidine (Sigma) for 5 min at RT. In negative controls, TdT was omitted from the nucleotide mixture. Several tissue sections were stained using a commercially available in situ apoptosis detection kit (ApoTag, Oncor, Gainthersburg, Md.), which involves TdT-mediated uptake of digoxigenin-labelled dUTP followed by incubation with a peroxidase-conjugated anti-digoxigenin antibody.

Statistical analysis

The number of the end-labelled cells in 5000 tumour cells was counted and its correlations with various prognostic factors were analysed statistically. Each prognostic factor comprised favourable (F) and unfavourable (UF) components: the extent of disease [stages I, II, and IV-S (F) and III and IV (UF)], the patient's age [under (F) and over (UF) 1 year], primary site of the tumour [extra-adrenal (F) and adrenal (UF)], occasion of the first diagnosis [mass screening (F) and clinical symptoms (UF)], and the Shimada classification [favourable histology (F) and unfavourable (UF) histology]. The results were expressed as mean± SD and difference between them was analysed using an unpaired Student's *t*-test. The level of significance was established at $P<0.05$.

Results

Morphological identification of apoptotic cells in neuroblastoma

Typically, the NBs examined consisted of closely packed elements resembling neuroblasts, which were disposed in clusters and sheets supported by a delicate fibrovascular stroma. According to the Shimada classification, 23 (66%) of the tumours had favourable and 12 (34%) unfavourable histology (Table 1). Individual tumour cells had hyperchromatic dense nuclei and a scanty rim of cytoplasm (Fig. 1a). In some areas, the tumour cells had undergone "karyorrhexis" [35], characterized by pyknotic or smashed nuclei and eosinophilic cytoplasms. There were no inflammatory exudates around the karyorrhectic cells (Fig 1a, b). Electron microscopy revealed that these cells had shrunk and had condensed nuclei and cytoplasm with rather compressed organelles. Each nucleus

Table 1 Clinicopathological features of neuroblastoma tumours (*ND* not done, as case prior to 1985 when mass screening programme started in Japan, *f* favourable histopathology, *uf* unfavourable histopathology)

Patient/ sample no	Age	Stage	Mass screening	Shimada class	*bcl*-2 staining[d]
1	3y1m	I	ND	f	<
2	6y0m	I	ND	f	0
3	5y2m	I	–	f	0
4	11m	I	+	f	<
5	11m	I	+	f	<
6	0m	I	–	f	<
7[a]	3y8m	I	–	f	≥
8	7m	II	ND	f	<
9[a,b,c]	8m	II	+	f	<
10[b,c]	3y7m	II	–	f	0
11	14y6m	II	–	f	≥
12	7m	II	+	f	<
13	7m	II	–	f	≥
14[b,c]	1y10m	II	ND	uf	<
15	9m	III	ND	f	<
16[a]	7m	III	+	f	<
17	2y7m	III	–	f	0
18[b,c]	9m	III	+	f	≥
19	4y2m	III	ND	uf	<
20	3y7m	III	ND	uf	0
21	3y3m	III	ND	uf	<
22[a,b]	5y4m	III	–	uf	≥
23	1y6m	IV	–	f	≥
24	1y2m	IV	–	f	≥
25	1y5m	IV	–	f	≥
26	7m	IV	+	f	≥
27	1y10m	IV	ND	uf	0
28	3y5m	IV	ND	uf	≥
29	1y3m	IV	ND	uf	≥
30	3y11m	IV	ND	uf	0
31	1y9m	IV	ND	uf	0
32	1m	IVS	–	f	<
33[a,b]	2m	IVS	–	f	<
34	4y9m	IVS	ND	uf	<
35	0m	IVS	ND	uf	<

[a] Fas antigen expressing tumour
[b] Tumour sample analysed by agarose gel electrophoresis
[c] DNA ladder-positive tumour
[d] <, ≥ less than, more than or equal to 50% of tumour cells positive for *bcl*-2; 0: no *bcl*-2 immunoreactivity

was irregular and had fragmented into a few pieces (Fig. 1c). These changes closely resembled the characteristics of apoptotic cells [20], suggesting that "karyorrhexis" represented apoptotic tumour cell death morphologically.

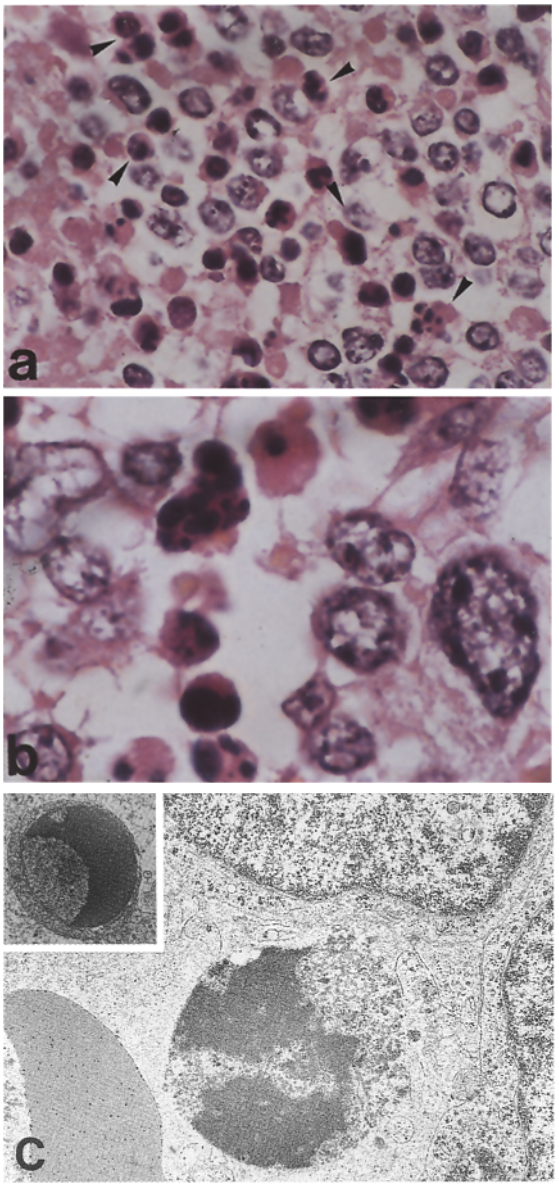
DNA Fragmentation in Neuroblastoma

Fragmentation of the DNA into a ladder of regular subunits, indicative of internucleosomal cleavage, was detected in four of six tumours (Fig. 2, lanes 4–7). It was noteworthy that two of the four tumours showing DNA ladders were detected by mass screening (patients 9 and 18, lanes 4 and 5). The ladders were not attributed to apoptosis induced by surgical removal of the tissues: they did not occur in the other two NBs (lanes 2 and 3), though these had been handled in an identical manner to the ladder-positive tumours.

We examined the DNA fragmentation in various cases by performing ISNEL. Of the 35 tumour samples analysed, 26 (74%) contained cells that incorporated biotinylated dUTP into their nuclei. The labelled nuclei comprised small roundish pieces that were scattered or occasionally gathered together (Fig. 3a, b). This was consistent with morphology and distribution of the karyorrhectic cells observed in paraffin sections (Fig. 1a, b), supporting our initial idea that karyorrhexis represented apoptosis. No ISNEL staining was observed in viable tumour tissues and some apoptotic cells and bodies (Fig. 3b). These results were reproducible when the detection system was replaced by the ApoTag peroxidase kit (data not shown). The labelled apoptotic cell counts ranged from 5 to 62 per 5000 tumour cells (mean 15.0, SD 14.5). However, there were no significant correlations between these counts and the clinicopathological prognostic factors of the patients (Table 2).

Expression of bcl-2 and Fas antigen in neuroblastoma

The 35 specimens examined included 27 that were *bcl*-2-positive to varying degrees, while 8 lacked immunoreactivity. In the latter, even the infiltrating lymphocytes used as internal positive controls failed to stain and these samples were excluded from the subsequent evaluation. The tumour specimens showed focal or diffuse staining patterns with nests of *bcl*-2-immunoreactive cells (Fig. 4a). Those cells were small- to medium-sized with staining confined to the cytoplasm (Fig. 4a, inset), the known localization of *bcl*-2 [14]. No nuclear staining was seen in any of the tumour specimens analysed. Notably, *bcl*-2 was hardly detected in areas composed of karyorrhectic, or apoptotic, cells (Fig. 4b) or in roundish pyknotic cells and surrounding tumour cells (Fig. 4c). The proportion of *bcl*-2-positive cells varied from specimen to specimen and ranged from 2% to 90%. Nine tumours expressed abundant *bcl*-2 (*bcl*-2/+50: ≥50% positive cells) and 18 contained less *bcl*-2 (*bcl*-2/–50: <50% positive cells), 14 of which expressed very little *bcl*-2 (2–20% positive cells). The *bcl*-2/–50 tumours were found more frequently in specimens from patients with stage IV-S disease (4 of 4 specimens: 100% *bcl*-2/–50) and favourable disease stages (I, II and IV-S; 12 of 15: 80%) than in specimens from patients with poor disease stages (III and IV; 4 of 12: 33%, Table 3).A similar distinction between NBs detected by mass screening (5 of 7 specimens: 71% *bcl*-2/–50) and clinical symptoms (3 of 10: 30%) during the same period was apparent (Table 3). Finally, frozen sections from 5 NBs were immunostained with a monoclonal anti-Fas antibody. Almost all tumour cells were Fas antigen-positive. The staining appeared as fine dots on the cell membranes, the known localization of the Fas receptor (Fig. 4d). There were no differences between the proportions of Fas-positive cells among the specimens analysed (90–95%). Virtually no Fas antigen was detect-



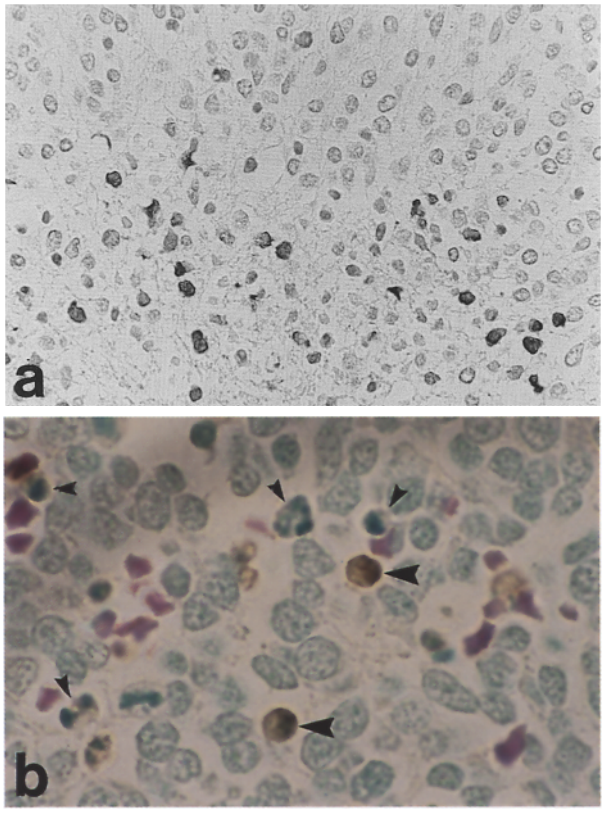


Fig. 3 a, b Detection of DNA fragmentation by ISNEL. Apoptotic nuclei incorporating biotinylated dUTP are evident (**a** *lower part* ×400, **b** large *arrowheads* ×1000; methyl green counterstain). Note that some apoptotic cells are left unstained with ISNEL (**b** *small arrowheads*)

Fig. 1 a–c Apoptosis in neuroblastoma. **a** Neuroblastoma with numerous karyorrhexes (*arrowheads*). HE, ×400. **b** High-power view of the karyorrectic cells. The karyorrhectic cells are characterized by smashed nuclei and eosinophilic cytoplasm. HE, ×1000. **c** Electron microscopy of a karyorrhectic, or apoptotic, cell (×8000) and an apoptotic body (*inset*; ×10,000). Note the marked condensation and fragmentation of the nucleus. Nucleus in the apoptotic body abuts on the nuclear envelope

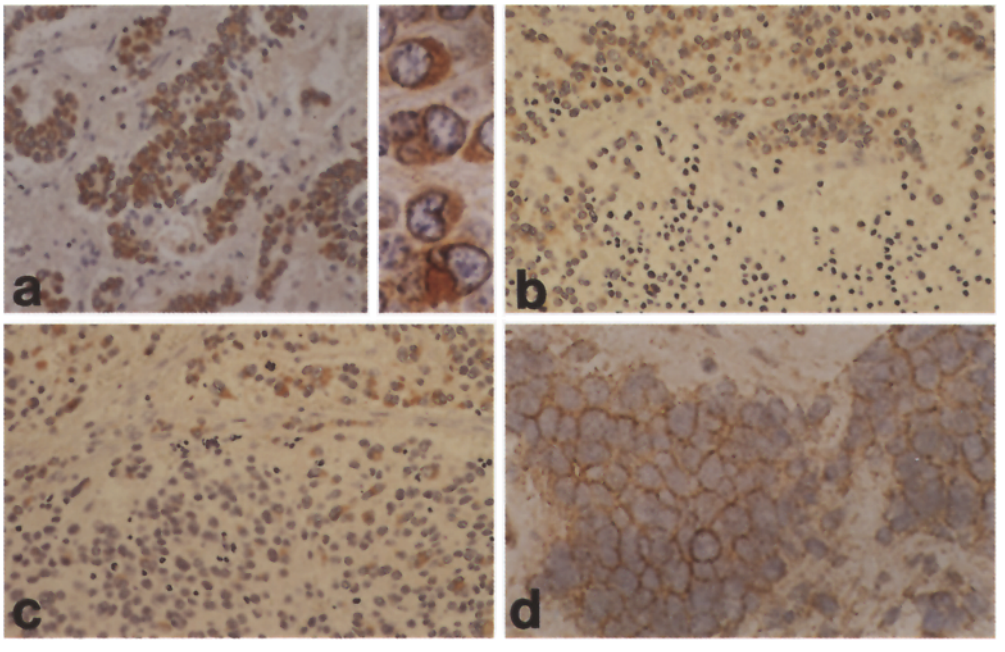


Fig. 4 a–d Immunoperoxidase staining of neuroblastoma tumour specimens. **a** Expression of *bcl*-2 in neuroblastoma cells (paraffin section, ×400, *inset* ×1000); *bcl*-2 staining is localized in the cytoplasm. **b** Loss of *bcl*-2 in apoptotic and pyknotic cells (paraffin section, ×400). **c** Loss of *bcl*-2 in tumour cells intermingled with apoptotic cells (paraffin section, ×400). Compare *upper* (*bcl*-2-positive) and *lower* (*bcl*-2 negative or deficient) *parts* of **a–c**. **d** Expression of Fas antigen in neuroblastoma cells (frozen section, ×400). Almost all the tumour cells express Fas antigen, visible as fine dots, on their cell membranes

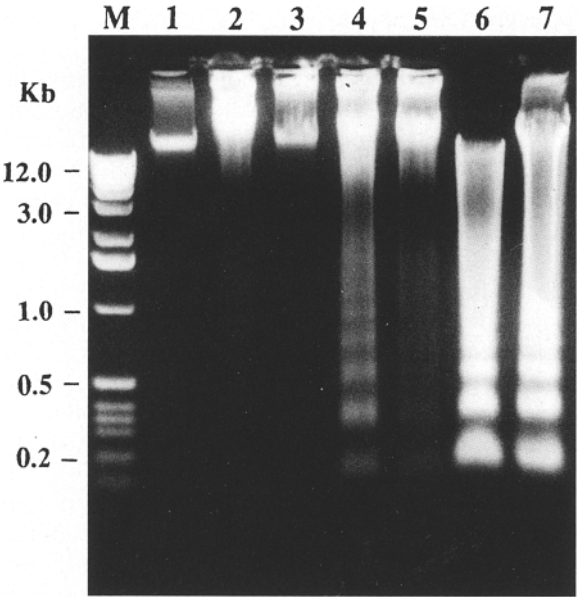


Fig. 2 Fragmentation of DNA in neuroblastoma. The tumour DNA was separated by an agarose gel electrophoresis. Lane 1 control liver, lanes 2–7 neuroblastomas. Characteristic DNA ladders are present in lanes 4–7. *M* Molecular weight marker (1-kb ladder)

ed in karyorrhectic-apoptotic cells and non-tumour components, including infiltrating lymphocytes, fibroblasts and endothelial cells.

Discussion

Apoptosis plays an important part in developmental biology, autoimmune disease, toxic effects leading to cell damage and tumour growth [6, 20, 21]. In a tumour, a balance between cell proliferation and apoptotic cell death is a crucial determinant of its net growth rate [3, 38]. Accordingly, apoptosis may lead to tumour regression when its extent exceeds that of cell proliferation [12, 19, 36]. The immunological and maturation hypotheses that have been advanced to explain disappearance of stage IV-S tumour [28] are not well supported, and the idea that apoptosis produces spontaneous NB regression seems plausible. To examine this possibility, we first examined whether NB actually underwent apoptosis in vivo. Microscopically, a number of NB tumour cells were found to show typical apoptotic changes, including cell shrinkage, condensed and fragmented nuclei, eosinophilic cytoplasm and lack of inflammatory response (Fig. 1) [20, 29]. Biochemical analyses provided further support for this observation by demonstrating DNA fragmentation. Gel electrophoresis resulted in the appearance of characteristic DNA ladders in four of six tumours, and in situ nick end-labelling demonstrated nuclear incorporation of dUTP in 26 of 35 (74%) tumour specimens (Fig. 3). Consequently, these present results provide the first definite evidence that apoptosis occurs in primary NB tissues.

We observed that the morphology and distribution of the apoptotic cells were virtually identical to those of the karyorrhectic cells (Figs. 1, 3). Karyorrhexis denotes broken nuclei and is a prognostic factor determinant in the Shimada classification [35]. In view of the difficulty in distinguishing karyorrhexis from mitosis, this classification involves using the sum of both counts in 5000 tumour cells as a mitosis karyorrhexis index (MKI) to divide patients with NB into subgroups with favourable (low MKI) and unfavourable (high MKI) prognosis. Recently, the apoptosis/mitosis count (A/M) ratio was shown, both in vitro and in vivo, to correlate very well with the net tumour growth rate [3]. Since apoptosis has a pivotal role in regulating the growth rate, a tumour with a high A/M ratio develops slowly and vice versa [3]. Accordingly, if our premise that karyorrhexis reflects apoptosis is correct, the karyorrhexis/mitosis count (K/M) ratio would be a more practical variable than the

Table 3 Association of *bcl*-2 deficiency with clinical stage, method of disease detection and histopathology in neuroblastoma

	Total no. of patients	*bcl*-2/-50 tumour[a] (absolute no.)	Frequency (%)
Clinical stage			
Stage IV-S	4	4	100.0
Stage I, II, IV-S	15	12	80.0
Stage III and IV	12	4	33.3
Disease detection			
Mass screening	7	5	71.4
Clinical symptoms	10	3	30.0
Shimada classification			
Favourable histology	19	11	57.9
Unfavourable histology	8	5	62.5

[a] Fewer than 50% of tumour cells express *bcl*-2.

Table 2 Number of nick end-labelled cells in neuroblastoma cases with favourable and unfavourable prognostic factors

Prognostic factor[a]	Positive cells/5000 tumour cells (mean±SD)				
	Age	Disease detection	Original site	Stage	Shimada class
Favourable	17.1±17.1 (n=14)	18.7±21.3 (n=7)	12.4±13.2 (n=14)	11.0±15.4 (n=18)	13.4±15.4 (n=23)
Unfavourable	13.3±13.1 (n=21)	13.9±12.8 (n=13)	16.0±15.5 (n=21)	18.8±12.7 (n=17)	17.5±13.6 (n=12)

[a] See Materials and methods for detailed components. No significant difference in the labelled cell counts is present between the two groups in any category (Student's t-test, P>0.05) .

MKI for estimating the growth rate of NB and predicting the prognosis of the patient. Careful light microscopic observation enables apoptotic and karyorrhectic figures to be distinguished from mitotic cells [1, 4] (H. Koizumi, unpublished work), and a new histological grading system for NBs requires *only* the mitotic figure count as a tumour risk indicator [18]. In order to determine which nuclear variable correlates most closely with the clinical outcome of the patient, a retrospective study comparing the K/M ratio, MKI (M+K) and plain mitotic count (M) [18] is currently under way in our laboratory.

It remains to be determined whether apoptosis is important in spontaneous regression of NB. Initially, we predicted that a regression-prone tumour in stage IV-S or detected by mass screening would undergo extensive apoptosis, as implied by several studies [12, 19, 36]. In fact, two of the four tumours that displayed electrophoretic DNA ladders were found during mass screening (Fig. 2, lanes 4 and 5), suggesting that extensive apoptosis occurs in this type of NB. However, the nick end-labelling results were conflicting: there was no significant difference between the labelling counts of the NBs prone to regression and the others (Table 2). Similarly, the labelling counts of the ladder-negative and -positive tumours were almost identical (data not shown), implying that nick end-labelling is less sensitive than gel electrophoresis for the detection of DNA fragmentation. This is compatible with our observation that a number of karyorrhectic-apoptotic cells were left unstained after labelling (Fig. 3b). The labelling sensitivity did not improve when we replaced the detection system for nick ends by one that utilized a digoxigenin-peroxidase composite (ApoTag; data not shown). These results were not attributed to insufficient predigestion of the specimens with protease, as the digestion time was optimized in each sample using DNase-treated positive control (see Materials and methods). Therefore, this insensitive labelling method probably resulted in the detection of a limited number of apoptotic cells, and the consequent counts may have been too low (15±14.5 counts per 5000 tumour cells) for valid statistical comparison. Nevertheless, it may still be possible that some of the unlabelled karyorrhectic cells do not represent apoptosis in NB, as intimated by Manjo and Joris [22]. Although *bcl*-2 immunostaining raises the possibility that NBs prone to spontaneous regression may be susceptible to apoptosis, a conclusive demonstration of the direct involvement of apoptosis in tumour regression awaits the development of a more sensitive nick end-labelling technique, DNA gel electrophoresis of various tumours and/or analysis of biopsy specimens taken from NBs in the process of spontaneous regression.

The *bcl*-2 and Fas antigen immunostaining results are fascinating and provide valuable information on the mechanism that regulates apoptosis in NB. Originally, *bcl*-2 was thought to be a proto-oncogene, and it was later shown to inhibit apoptosis specifically [14]. The Fas antigen, or APO-1, was found to be a counterpart of antibody that induced apoptosis and is believed to be a specific receptor for apoptosis [16, 37]. In NB, the loss of *bcl*-2-mediated apoptotic suppression appears to play an important part in the occurrence of apoptosis, as virtually no *bcl*-2 was detected in karyorrhectic-apoptotic cells in any of the tumour specimens analysed (Fig. 4b). Similarly, no *bcl*-2 was detected in the roundish pyknotic cells surrounding karyorrhexis, implying that these cells may be destined for apoptosis. Furthermore, we found that NBs prone to spontaneous regression (stage IV-S and those detected by mass screening) and those from patients with favourable disease stages (I, II and IV-S) rarely expressed *bcl*-2 protein (*bcl*-2/50 tumours, Table 2; Fig. 4c), suggesting that these types of NB may be susceptible to apoptosis. This agrees with some other work, which has indicated a correlation between enhanced *bcl*-2 expression and advanced disease stages [8, 30]. Clearly, the lack of *bcl*-2 is insufficient in itself to cause apoptosis and the tumour cells may require (an) additional signal(s) to turn on their apoptotic programme. In this regard, it is interesting to note that most NB cells (ca. 95%) readily expressed Fas receptors on their cell surfaces (Fig. 4d). Therefore, some extrinsic apoptotic signal(s) transmitted via the Fas receptor may provide the "second hit" on *bcl*-2 lacking tumour cells that eventually induces apoptosis. Alternatively, the second signal may be intrinsic, in which case an as yet unidentified cytogenetic event may lead to programmed cell death of the cells deficient in *bcl*-2.

To date, several apoptosis regulators have been found in NB cell lines, including tissue transglutaminase [26], retinoic acid [27] and nerve growth factor (NGF) [17], which functions via the low-affinity NGF receptor ($p75^{NGFR}$) [29]. The identification of a factor(s) that initiates apoptosis physiologically is important for elucidation of the mechanism(s) underlying spontaneous regression of primary NB. Moreover, the factor(s) may have potential application as (a) novel therapeutic agent(s) for NB, together with antisense oligonucleotide against *bcl*-2 mRNA and functional antibody against the Fas antigen.

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