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Original Paper

Interleukin-1β Converting Enzyme (ICE) is Preferentially Expressed in Neuroblastomas with Favourable Prognosis

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To determine whether interleukin-1β converting enzyme (ICE) plays a role in the programmed cell death of neuroblastoma, we studied ICE expression in primary tumours. In patients in stages I, II and IVS, ICE mRNA was detected in 22 of 32 (69%) tumours, while only 5 of 26 (19%) tumours expressed ICE in stages III and IV ($P < 0.001$). ICE mRNA was expressed in 27 of 47 (57%) tumours without *MYCN* amplification, but it was not detected in any tumours with *MYCN* amplification ($P < 0.01$). Immunohistochemically, the cytoplasm was stained in all 15 neuroblastomas examined. The nuclei were stained in 12 neuroblastomas without *MYCN* amplification, whereas only 1 of 3 tumours with *MYCN* amplification had positive staining in the nuclei. In ganglioneuromas, high levels of ICE mRNA were expressed, but immunostaining showed that the protease expression was confined to the cytoplasm. These observations suggest that ICE may be associated with the spontaneous regression often seen in favourable neuroblastomas and that localisation of ICE protease in the cell may be important for the cell death pathway. Double staining for ICE and TUNEL showed that they were co-localised in some nuclei, but the distribution of ICE protease expression was not necessarily the same as that of DNA fragmentation, suggesting that the protease expression probably preceded DNA fragmentation during the apoptotic process. ICE may play an important role in regulating the apoptotic process of neuroblastoma. © **1997 Elsevier Science Ltd.**

Key words: interleukin-1β converting enzyme (ICE), apoptosis, neuroblastoma
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INTRODUCTION

NEUROBLASTOMA IS a paediatric solid tumour which originates in the adrenal and the sympathetic ganglia. It is clinically and biologically heterogeneous, with some tumours regressing spontaneously whilst others progress. Most neuroblastomas with a favourable prognosis express high levels of a high-affinity nerve growth factor (NGF) receptor gene, *trk*-A, and differentiate in response to NGF [1, 2]. Depletion of NGF induces cell death of such neuroblastomas in primary culture, and this phenomenon may occur *in vivo* [2]. We previously observed that massive apoptosis occurred in some neuroblastomas with favourable characteristics [3]. It has also been found that the *bcl-2* proto-oncogene product may regulate apoptosis in such regressing tumour cells [3, 4].

Interleukin-1β converting enzyme (ICE) is a mammalian homologue of CED-3 which is required for programmed cell death in the nematode *Caenorhabditis elegans* [5]. A recent study revealed that an inhibitor of ICE prevented neuronal cell death induced by NGF deprivation, suggesting that ICE may participate in the neuronal cell death process in vertebrates [6]. In this study, we studied ICE expression in primary neuroblastomas to determine whether ICE plays a role in the programmed cell death of this tumour.

MATERIALS AND METHODS

Tissue samples

Tumour specimens were obtained at surgery from 58 neuroblastoma and 2 ganglioneuroma patients and were

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subjected to Northern blotting. The patients were treated at the Children's Hospital of Philadelphia and the St. Louis Children's Hospital in the U.S.A. and the Department of Paediatric Surgery, Chiba University, the Department of Paediatrics, Kagoshima University, the Department of Paediatric Surgery, Kumamoto University, and the Department of Paediatric Surgery, Hiroshima University, in Japan during the past 5 years. The stages of the neuroblastomas were stage I in 17 cases, stage II in 6, stage IVS in 9, stage III in 13 and stage IV in 13 patients according to the staging system by Evans and associates. Immunohistochemical studies were done with samples obtained at Gunma Children's Medical Centre during the past 5 years from 15 neuroblastoma and 2 ganglioneuroma patients. The stages of the neuroblastomas were stage II in 4, stage IVS in 1, stage III in 6 and stage IV in 4 patients.

RNA extraction and Northern blotting

Total RNA was extracted from frozen samples of tumour tissues and was subjected to Northern blot analysis as described previously [7]. Briefly, 25 μg of each RNA was resolved on 1% agarose-formaldehyde gels and transferred to a nylon membrane by blotting. Blots were hybridised with ICE cDNA probe and were exposed to X-ray films to obtain autoradiographs. The ICE cDNA probe was prepared by one of the authors (M.S.).

Immunostaining of ICE

Haematoxylin and eosin sections were examined in all specimens and a representative block was chosen from each of the tumours. Sections from formalin-fixed, paraffin-embedded samples were deparaffinised, rehydrated and processed for staining. A rabbit polyclonal antibody to 10 kDa (p10) subunits of ICE (Santa Cruz Biotechnology, Inc., Santa Cruz, California, U.S.A.) was applied at a dilution of 1:100 and detected by the streptavidin–biotin (SAB) method. Because ICE expression in a section was not homogeneous, standards for qualification were used. When positive cells were observed, ICE expression was described as '+1' and it was described as '+2' if strongly positive cells were observed. When only weakly positive cells were observed, the expression was described as '±'.

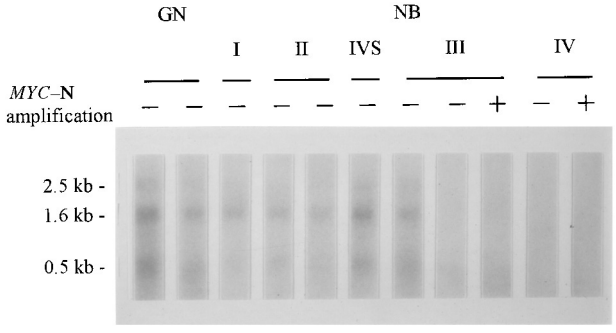

Figure 1. Northern blot analysis of neuroblastomas and ganglioneuromas. Relatively high levels of ICE transcript were seen in samples of ganglioneuromas and a IVS neuroblastoma. ICE expression was not detectable in tumours in the advanced stages with or without *MYC*N amplification. GN, ganglioneuroma; NB, neuroblastoma; +, *MYC*N amplification > 10 copies; –, no *MYC*N amplification.

Identification of apoptotic cells (TUNEL method) and double labelling for ICE and TUNEL

The *in situ* visualisation of apoptotic cells was done by the method described by Gavrieli and associates [8]. Briefly, the exposure of nuclear DNA on histological sections was completed by incubation with 20 μg/ml proteinase K. Terminal deoxynucleotidyl transferase (TdT) (Life Sciences, St Petersburg, Florida, U.S.A.) was used to incorporate biotinylated deoxyuridine triphosphate (dUTP) (Enzo Diagnostics, New York, U.S.A.) at sites of DNA breaks. Double labelling for ICE and TUNEL with fluorescence was done according to the method described in our previous paper [3]. In this study, conjugates of ICE and anti-ICE antibody were detected with a fluorescein-5-isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Organon Teknika Corporation, Durham, North Carolina, U.S.A.).

Statistical analysis was done by the χ^2 test.

RESULTS

ICE mRNA expression

Fifty-eight primary neuroblastomas were analysed by Northern blotting to detect ICE transcript (Figure 1). In patients in stages I, II and IVS, ICE mRNA was detected in 22 of 32 (69%) tumours, while 5 of 26 (19%) tumours expressed ICE in stages III and IV ($P < 0.001$). ICE mRNA was expressed in 27 of 47 (57%) tumours without *MYC*N amplification, but it was not detected in any tumours with *MYC*N amplification ($P < 0.01$).

Immunostaining of ICE

The results of immunostaining of ICE protease in 15 neuroblastomas are summarised in Table 1. The cytoplasm was stained in all 15 neuroblastomas. The nuclei were stained in 12 neuroblastomas without *MYC*N amplification, whereas only 1 of 3 tumours with *MYC*N amplification had positive staining in the nuclei (Figure 2a). Two neuroblastomas with *MYC*N amplification had negative staining in the nuclei (Figure 2b). ICE protease seemed to be expressed in the nuclei more strongly in favourable neuroblastomas than in aggressive tumours.

Table 1. *Immunostaining of interleukin-1β converting enzyme (ICE) protease in* 15 *neuroblastomas*

Case	Age (months)	Stage	*MYC*N (copy)	ICE protease	
				Nucleus	Cytoplasm
1	6	II	1	+	±
2	7	II	1	+	+
3	8	II	1	+	+
4	9	II	1	+	+
5	9	IVS	1	++	±
6	2	III	1	+	±
7	4	III	1	++	+
8	7	III	1	+	+
9	9	III	1	+	+
10	11	III	1	+	++
11	22	III	40	++	+
12	8	IV	1	+	+
13	19	IV	140	–	+
14	50	IV	250	–	±
15	56	IV	1	+	+

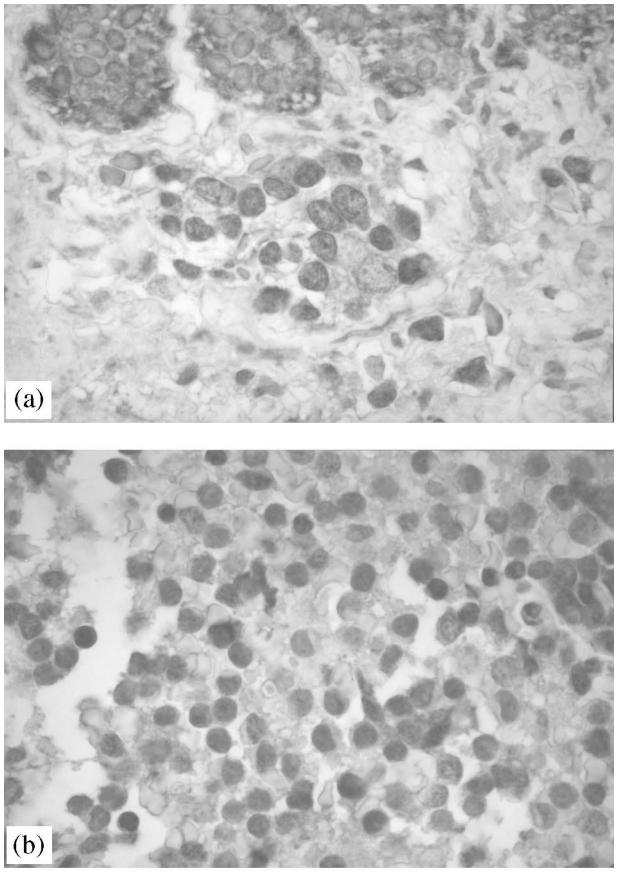

Figure 2. ICE immunostaining of an (a) IVS neuroblastoma originating in the adrenal gland detected by screening. Neuroblastoma cells showed positive staining for ICE, in both the nuclei and the cytoplasm, but strong positivity was observed in the nuclei. (b) A stage IV unfavourable neuroblastoma with *MYCN* amplification. ICE was expressed weakly in the cytoplasm, and most cells do not show ICE expression in the nuclei. (Immunoperoxidase, original magnification ×400.)

In ganglioneuromas, ICE expression was observed only in the cytoplasm of mature ganglion cells. No staining was observed in the normal adrenal medulla.

Double staining for ICE and TUNEL with fluorescence

Double staining for ICE and TUNEL was done in 2 cases whose specimen was positive for both ICE and TUNEL (data not shown). Both ICE and TUNEL were positive in some nuclei, but the distribution of ICE protease expression was not necessarily the same as that of DNA fragmentation.

DISCUSSION

Interleukin-1β converting enzyme (ICE) is an oligomeric enzyme with 10 kDa (p10) and 20 kDa (p20) subunits that are derived from a common 45 kDa precursor [9]. ICE functions as a cysteine protease that cleaves 33 kDa interleukin-1β (IL-1β) precursor into 17.5 kDa biologically active IL-1β [10]. Overexpression of ICE induced programmed cell death, which was suppressed by the product of the crmA gene, a specific inhibitor of ICE [6, 11]. A recent study demonstrated that the ICE family proteases are components of the mammalian cell death pathway [12]. Active forms of the proteases cause cell death by accessing cytoplasmic and nuclear substrates such as poly (ADP-ribose) polymerase (PARP). It was also demonstrated that bcl-2 may function upstream and inhibit apoptosis by blocking the cell death signal that activates the proteases [12].

In this study, we observed that ICE mRNA was more frequently or preferentially expressed in neuroblastomas in stages I, II and IVS and more frequently expressed in tumours without *MYCN* amplification. Immunohistochemically ICE was expressed in the cytoplasm and the nuclei in neuroblastomas, but strong positivity tended to be observed in the nuclei in favourable neuroblastomas. These observations suggest that ICE may play an important role in regulating the apoptotic process of neuroblastoma and that ICE may be associated with the spontaneous regression often seen in favourable neuroblastomas. In ganglioneuromas, high levels of ICE mRNA were expressed, but immunostaining showed that protease expression was confined to the cytoplasm. Localisation of ICE protease in the cell may have some biological meaning.

Double staining for ICE and TUNEL showed that they were co-localised in the nuclei of some tumour cells, but the distribution of ICE protease expression and that of DNA fragmentation did not always overlap. This may be explained if the protease expression precedes DNA fragmentation during the apoptotic process. In conclusion, ICE may play an important role in regulating the programmed cell death of neuroblastoma.

1. Nakagawara A, Arima-Nakagawara M, Azar CG, Scavarda NJ, Brodeur GM. Inverse relationship between trk expression and N-myc amplification in human neuroblastoma. *Cancer Res* 1992, **52**, 1364–1368.
2. Nakagawara A, Arima-Nakagawara M, Scavarda NJ, *et al.* Association between high levels of expression of the TRK gene and favourable outcome in human neuroblastoma. *N Engl J Med* 1993, **328**, 847–854.
3. Ikeda H, Hirato J, Akami M, *et al.* Massive apoptosis detected by *in situ* DNA nick end labelling in neuroblastoma. *Am J Surg Pathol* 1996, **20**, 649–655.
4. Ikeda H, Hirato J, Akami M, *et al.* Bcl-2 oncoprotein expression and apoptosis in neuroblastoma. *J Pediatr Surg* 1995, **30**, 805–808.
5. Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1β converting enzyme. *Cell* 1993, **75**, 641–652.
6. Gagliardini V, Fernandez P, Lee RKK, *et al.* Prevention of vertebrate neuronal death by the crmA gene. *Science* 1994, **263**, 826–828.
7. Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM. Expression and function of TRK-B and BDNF in human neuroblastoma. *Mol Cell Biol* 1994, **14**, 759–767.
8. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992, **119**, 493–501.
9. Gu Y, Wu J, Faucheu C, *et al.* Interleukin-1β converting enzyme requires oligomerization for activity of processed forms *in vivo*. *EMBO J* 1995, **14**, 1923–1931.
10. Thornberry NA, Bull HG, Calaycay JR, *et al.* A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. *Nature* 1992, **356**, 768–774.
11. Miura M, Zhu H, Rotello R, Hartweig EA, Yuan J. Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene ced-3. *Cell* 1993, **19**, 653–660.
12. Chinnaiyan AM, Orth K, O'Rourke K, *et al.* Molecular ordering of the cell death pathway. *J Biol Chem* 1996, **271**, 4573–4576.