Pergamon

European Journal of Cancer, Vol. 33, No. 12, pp. 2007–2010, 1997

Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: S0959-8049(97)00294-3

Original Paper

Betulinic Acid Induces Apoptosis in Human Neuroblastoma Cell Lines

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Neuroblastoma has long been recognized to show spontaneous regression during fetal development and in the majority of stage 4s infants < 1 year of age with disseminated disease. Stage 4s disease regresses with no chemotherapy in 50% of the patients. The mechanism by which this occurs is not understood but may be programmed cell death or apoptosis. Betulinic acid (BA) has been reported to induce apoptosis in human melanoma with *in vitro* and *in vivo* model systems. Melanoma, like neuroblastoma, is derived from the neural crest cell. We hypothesised that neuroblastoma cells have the machinery for programmed cell death and that apoptosis could be induced by betulinic acid. Nine human neuroblastoma cell lines were treated *in vitro* with BA at concentrations of 0–20 μg/ml for 0–6 days. Profound morphological changes were noted within 3 days. Cells withdrew their axonic-like extensions, became non-adherent and condensed into irregular dense spheroids typical of apoptotic cell death (ED_{50} = 14–17 μg/ml). DNA fragmentation analysis showed ladder formation in the 100–1200 bp region in 3/3 neuroblastoma cell lines treated with BA for 24–72 h. Thus, apparently BA does induce AP in neuroblastoma *in vitro*. This model will be utilised to investigate the role of apoptosis-related genes in neuroblastoma proliferation and to determine the therapeutic efficacy of BA in neuroblastoma *in vivo*. © **1997 Elsevier Science Ltd.**

Key words: neuroblastoma, apoptosis, betulinic acid
Eur J Cancer, Vol. 33, No. 12, pp. 2007–2010, 1997

INTRODUCTION

Apoptosis is the cellular process whereby death occurs in a selective and developmentally-regulated process. The process is involved in embryological moulding, normal cell turnover, immune regulation and hormone-dependent atrophy [1]. Tumour cell death can occur by necrosis or apoptosis and these processes are separate and distinct. Necrosis occurs secondary to hypoxia or exposure to exogenous cytotoxic agents. Programmed cell death (PCD) results from signals generated within the eukaryotic organism. The induction, or triggering, of PCD is commonly developmentally regulated and results in the removal of unnecessary, overabundant or superfluous cells. Apoptosis has euphemistically been named 'altruist suicide' in that the cells that receive the signals to die obediently do so.

Neuroblastoma is the most common solid tumour in early childhood [2] and develops in the adrenal medulla or sympathetic ganglia. While introduction of aggressive combination chemotherapy protocols has increased the survival rates for many forms of childhood neoplasms including acute leukaemia, Wilms' tumour and osteosarcoma, advanced stage neuroblastoma remains intractable to chemotherapy. Neuroblastoma has a particularly poor prognosis (< 30% survival) in patients older than 2 years of age at diagnosis, advanced stage disease and/or disease characterised by *MYCN* gene amplification [3, 4]. *MYCN* is an oncogene which has a developmentally-dependent pattern of expression limited to embryonic and fetal development. Expression of *MYCN* is not detectable in mature normal tissues [5]. Seeger and associates recognized that *MYCN* amplification in neuroblastoma tumour cells confers a poor prognosis independent of age and stage [4].

By contrast, low-stage neuroblastoma patients diagnosed at before 2 years of age and those tumours with a single copy *MYCN* are cured more than 75% of the time [6]. Furthermore, there is also a unique advanced stage neuroblastoma which occurs in infants under 1 year of age, stage 4S, that

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shows a remarkably high spontaneous regression rate, nearly 100 times that of any other human cancer [6]. This suggests that at least one form of neuroblastoma may exhibit delayed onset of apoptosis by a mechanism that remains to be defined.

Recently, the expression of several genes, including *MYC*N, has been associated with the induction or suppression of apoptosis. Expression of *bcl-2* (and the related gene *bcl-x*) correlates with suppression of apoptosis [7]. Overexpression of *bcl-2* and *bcl-x* correlates with overexpression of *MYC*N [8, 9] and modulates chemotherapy-induced apoptosis [10], possibly through interaction with the multidrug resistance-associated protein gene *MRP* [11]. *MRP* is expressed in poor-prognosis neuroblastoma which also exhibits overexpression of *MYC*N. While *bcl-2* suppresses apoptosis, its heterodimeric partner, *bax*, accelerates apoptosis [12] and its homologue *bak* counteracts the *bcl-2* suppressed apoptosis machinery. Both *bcl-2* and *myc* appear to be under the regulation of the tumour suppressor *p53* [13].

Several anticancer drugs appear to act by induction of apoptosis. Recently, the pentacyclic triterpene, betulinic acid (BA), was reported to induce apoptosis in cultured melanoma cells and cause nearly complete regression of human melanoma tumours carried in athymic mice [14]. Few previous reports of the tumour-inhibitory properties of BA are available and suggest only marginal effectiveness. The ED_{50} for BA with melanoma cells in culture was 1.1–4.8 μg/ml. Betulinic acid exhibited no toxicity in athymic mice at 500 mg/kg body weight (approximately 35 g per treatment for the average person). An ideal antineoplastic agent would have little non-specific toxicity and high specific toxicity for neoplasms. Betulinic acid may be such an agent if it triggers selective PCD with tumour cells.

We hypothesised that neuroblastoma cells have functional PCD machinery and that apoptosis can be induced by BA. Our hypothesis is based upon the fact that neuroblastoma and melanoma share a common neural crest cell origin; stage 4s neuroblastoma exhibits apparent spontaneous apoptotic regression; and betulinic acid induces apoptosis *in vitro* and *in vivo* in melanoma.

MATERIALS AND METHODS

Established human neuroblastoma cell lines were utilised in the current study. *MYC*N amplified (LAN-5; IMR-5; and NBL-W and the corresponding cell line NBL-WR, established at the time of relapse) and non-amplified (SKNSH and SHSY5Y) cell lines, as well as the single-copy *MYC*N cell line NBL-S [15] with increased *MYC*N protein expression were utilised. Finally, two antisense *MYC*N RNA expressing neuroblastoma cell lines (NBAS 5 and NBAS 6) derived from NBL-S cells and established by M.L.S., were also included in the study [16]. These cell lines are unique in that *MYC*N protein expression has been downregulated by 50%, resulting in decreased clonogenicity in soft agar. All cell lines were maintained in cell culture at 5% CO_2, 37°C, in RPMI 1640 media with 10% fetal calf serum and penicillin–streptomycin, amphotericin B and glutamine.

Light microscopy

1×10^5 neuroblastoma cells were plated into 24-well plates. Cells were treated with BA at 0, 1, 3, 6, 10 and 20 μg/ml of BA for 6 days. The effect of BA treatment on detachment and accumulation of free floating, non-adherent cells was recorded.

Sulphorhodamine B assay

ED_{50} values resulting from BA treatments were measured by the sulphorhodamine (SRB) dye liberation technique [17]. Four neuroblastoma cell lines (SKNSH, IMR-5, NBL-S and LAN-5) were treated with BA at concentrations of 0, 6 or 20 μg/ml. Cells were collected at 0, 6, 24 and 48 h after initiation of BA treatment. Briefly, cells were fixed with 50% trichloroacetic acid (at 4°C) and stained with 0.4% SRB in 1% acetic acid. The bound SRB was released with 0.1 M Tris base the absorbance measured at A_{570}. Results are reported as an 'inhibition rate' ([1−Treatment/Control] × 100%).

Propidium iodide assay

Detection of apoptotic cells was determined by the modified propidium iodide technique of Jacobson and associates [18]. Three neuroblastoma cell lines (SKNSH, IMR-5 and LAN-5) were treated with BA at 0, 6 or 20 μg/ml for 0, 6, 24 and 48 h and collected by standard technique. Briefly 5×10^5 cells were centrifuged at low speed (400 *g*) for 10 min. The cells were rinsed in KCl (75 mM) for 10 min. Cells were pelleted and treated twice with MeOH/AcH (3:1) for 15 min and pelleted. Cells were fixed onto slides and air-dried for 72 h. Slides were incubated with 0.05 μg/ml propidium iodide in phosphate-buffered saline (PBS) and 100 μg/ml RNase A for 30 min in the dark and visualised by fluorescent microscopy.

DNA fragmentation

Internucleosomal fragmentation forms integer multiples of 180–200 bp fragments [19]. A Ca^{2+}-dependent endonuclease cleaves internucleosomal linker DNA to the size of DNA wrapped around a single histone octamer. This degradation pattern can be observed as a DNA ladder using standard agarose gel electrophoresis. Three neuroblastoma cell lines (SKNSH, IMR-5 and LAN-5) were examined for internucleosomal fragmentation after treatment with BA at 20 μg/ml and collected at 0, 6, 24 and 48 h after initiation of BA treatment. The method of Ritke and associates was utilised to visualise DNA fragmentation [20]. Briefly, after *in vitro* treatment with BA, approximately 2×10^6 cells were pelleted by low-speed (~100 *g*) centrifugation for 5 min. The cells were washed with normal saline (0.85% NaCl), then solubilised for 1 h at 50°C in lysis buffer (20 μl/cell pellet; 50 mM tris-HCl, 10 mM EDTA, 0.5% sodium laurylsarcosine, 10 μg proteinase K, pH 8.0). RNase A was added (10 μl at 0.5 mg/ml) after initial lysis (1 h) and incubated for 1 h at 50°C. Proteolysis and RNA degradation was terminated by raising the sample temperature to 70°C for 5 min. Agarose gels (2% w/v) prepared and run with TBE buffer were used for DNA fragmentation visualisation. Samples were loaded into the wells by preparing 1% agarose plugs and were run at 40 V overnight (room temperature). The neuroblastoma cell lines were compared to HT1080 human sarcoma cells and Mel2 melanoma cells under the described conditions.

RESULTS

Light microscopy

Initial investigation of BA for cytotoxic action on human neuroblastoma cell lines showed classic apoptosis. A time course of 6 days with a concentration series of 1–20 μg/ml BA showed that all nine neuroblastoma cell lines tested were sensitive to the cytotoxic action of BA (Table 1). At the highest concentration tested (20 μg/ml), all neuroblastoma

cells were killed within 72 h. After treatment with BA, neuroblastoma cells undergoing apoptosis exhibited a characteristic appearance. Adherent monolayer forming cells retracted their axonal-like extensions and detached. The cells became spheroid with exvaginations (Figure 1a and b).

Table 1. *Betulinic acid cytotoxicity in neuroblastoma cell lines*

	Betulinic acid treatment (μg/ml)				
Cell lines	1	3	6	10	20
SKNSH	–	–	–/+	++	++++
SHSY5Y	–	–	–/+	+++	++++
IMR-5	–	–/+	++	++++	++++
LAN-5	–	+	++	+++	++++
NBL-S	–	–	+	+++	++++
NBAS5	–	–	+	+++	++++
NBAS6	–	–	+	+++	++++
NBL-W	–	–	+++	+++	++++
NBL-WR	–	–	+	++	++++
MEL-2	–	+/–	+	++	++++

Neuroblastoma cells (1×10^5) were plated into 24-well plates and were treated with BA in standard culture medium for 3 days. The effect of BA on detachment and accumulation of free floating, non-adherent cells was recorded. A six-point scale was used with (–) for no observable cytotoxicity, (+/–) some possible but not clear cut cytotoxicity, and a gradation from (+ to ++++) to indicate clearly observable cytotoxic effects with increasing numbers of invaginated, detached cells with increasing cell debris.

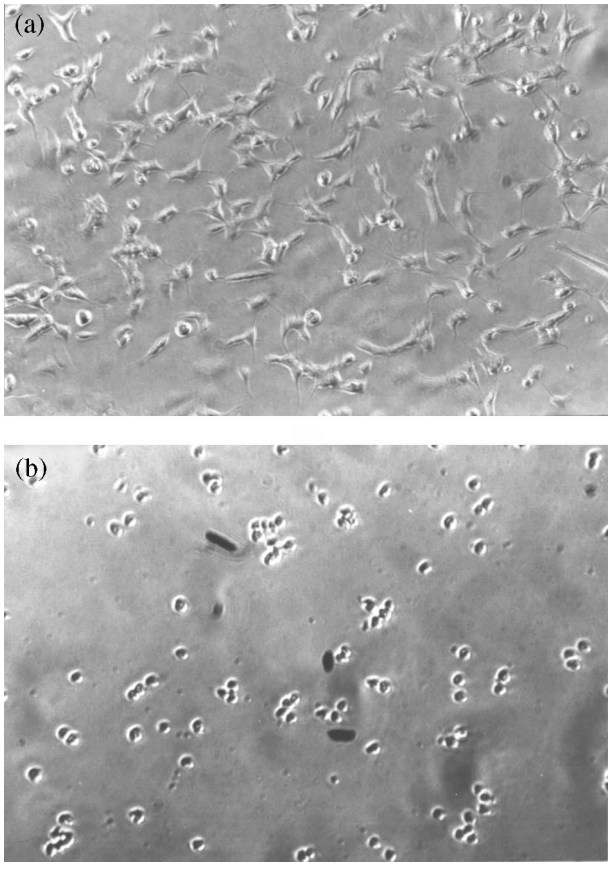

Figure 1. Light microscopy. 5×10^5 SKNSH human neuroblastoma cells were grown in RPMI with 10% fetal calf serum and additives in a 25 cm^3 plate. Cells were treated with BA at 20 μg/ml for 72 h. (a) Control untreated cells. (b) Treated cells.

Sulphorhodamine B assay

SRB quantitation showed increased inhibition rates for both increased concentration of BA and increased time of exposure to BA (data not shown). The ED_{50} was approximately 14–17 μg/ml for a BA treatment time of 48 h.

Propidium iodide

Three human neuroblastoma cell lines (SKNSH, IMR-5 and LAN-5) were treated with BA at concentrations of 0, 6 or

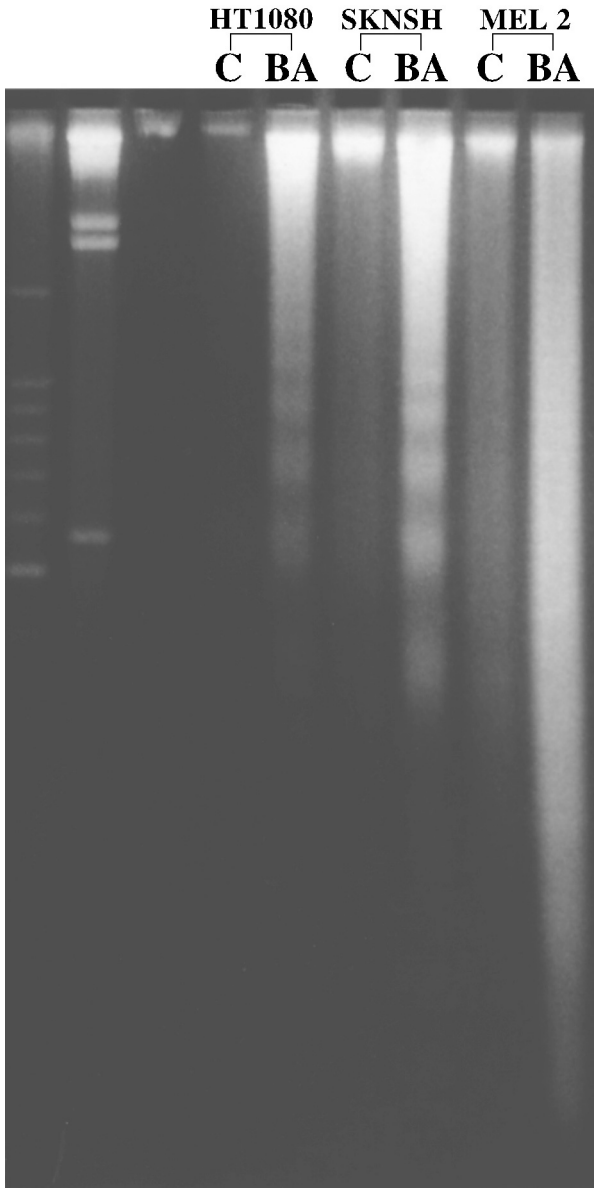

Figure 2. DNA fragmentation analysis. Three human tumour cell lines, HT1080 (sarcoma), SKNSH (neuroblastoma) and Mel-2 (melanoma), were examined for apoptosis-induced DNA fragmentation after treatment with BA at 20 μg/ml. DNA was isolated as described and separated on a 2% agarose gel electrophoresed with TBE buffer for 24 h at 40 V. DNA fragmentation is present in cells treated for 72 h (labelled BA). Low levels of smearing, possibly resulting from mechanical shearing, were evident in the control (untreated) cell samples (labelled C). Lane 1—1 kbp ladder; Lane 2—lambda marker.

20 μg/ml and cells were collected at 0 and 48 h after initiation of BA treatment. At 20 μg/ml, the nuclei of most of the neuroblastoma cells were disrupted and residual nuclear fragments appear consistent with apoptotic bodies (data not shown).

DNA fragmentation analysis

Based on microscopic observation, three cell lines were examined (SKNSH, IMR-5, LAN-5) for evidence of internucleosomal fragmentation. DNA fragmentation typical of apoptosis was evident after treatment with BA for 72 h at 20 μg/ml in all three cell lines. SKNSH showed the most distinct banding pattern (Figure 2).

DISCUSSION

Investigators have long recognised the very frequent spontaneous regression rate of neuroblastomas during both fetal development and in stage 4s disease. The mechanism by which this regression occurs is unknown but may depend on apoptosis. Pisha and associates recently reported on an inducible model system for apoptosis in melanoma by treatment with BA [14]. We hypothesised that the machinery for apoptosis was intact in neuroblastomas and could be induced by BA.

In the current study, the effect of BA treatment of nine human neuroblastoma cell lines was examined. Morphological and biochemical analysis suggests that BA appears to induce apoptosis in these neuroblastoma cells. Treatment with 10–20 μg/ml of BA resulted in >90% cell death at 72 h. Cytotoxicity studies demonstrated ED_{50} values in the range of 14–17 μg/ml at 48 h. Light microscopic observations revealed that axonic-like projections were retracted and propidium iodide staining revealed fragmentation of the nucleus after treatment with BA. Finally, molecular analysis of apoptosis was examined by DNA fragmentation and the DNA ladder characteristic of apoptosis was evident after treatment with BA on three different neuroblastoma cell lines.

The prognosis for advanced stage neuroblastoma in children older than 2 years of age remains dismal despite multimodal aggressive therapy with an overall survival rate of less than 30%. Further intensification of therapy may produce intolerable toxicities. Therefore, alternative pharmacological approaches which have minimal non-specific toxicities should be explored. Some anticancer agents, including vincristine, etoposide and cisplatin, induce apoptosis [8, 10]. Each of these agents is frequently utilised in current protocols for the treatment of advanced-stage neuroblastoma and all have significant non-specific toxicities. BA could be a useful anticancer agent because it appears to induce apoptosis in susceptible cells and to date has no recognised non-specific toxicities.

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