

Betulinic Acid Induces Apoptosis Through a Direct Effect on Mitochondria in Neuroectodermal Tumors

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Background and Procedure. We identified BetA as a new cytotoxic agent active against neuroectodermal tumor cells including neuroblastoma, medulloblastoma, glioblastoma and Ewing sarcoma cells, representing the most common solid tumors of childhood. **Results.** BetA induced apoptosis by a direct effect on mitochondria independent of accumulation of wild-type p53 protein and independent of death-inducing ligand/receptor systems such as CD95. Mitochondrial perturbations on treatment with BetA resulted in the release of soluble apoptogenic factors such as cytochrome c or AIF from mitochondria into the cytosol, where they induced activation of

caspases. Overexpression of the anti-apoptotic proteins Bcl-2 or Bcl-X_L that blocked loss of the mitochondrial membrane potential and cytochrome c release from mitochondria also conferred resistance to BetA. Most importantly, BetA exhibited potent antitumor activity on neuroblastoma cells resistant to CD95- or doxorubicin-triggered apoptosis and on primary tumor cells from patients with neuroectodermal tumors. **Conclusions.** Thus, BetA may be a promising new agent in the treatment of neuroectodermal tumors including neuroblastoma in vivo. *Med. Pediatr. Oncol.* 35:616–618, 2000. © 2000 Wiley-Liss, Inc.

Key words: betulinic acid; neuroblastoma; mitochondria; apoptosis; drugs

INTRODUCTION

For advanced neuroblastoma, chemotherapy is considered an indispensable treatment modality [1]. Cytotoxic drugs with different modes of action have been found to induce apoptosis in sensitive target cells [2–7]. Mitochondria are considered to play a critical role in the regulation of various apoptotic processes including drug-induced apoptosis [8]. A common step in apoptosis is postulated to involve loss of the mitochondrial membrane potential and hyperproduction of reactive oxygen species [8]. Cytochrome c is released from mitochondria into the cytosol, where it is involved in the activation of effector caspases such as caspase-3 [8].

Because resistance to chemotherapy is a major concern in pediatric oncology, identification of new anticancer agents may be crucial for more effective therapies. BetA, a pentacyclic triterpene derived from white birch trees, has been identified as a new cytotoxic compound active on melanoma and neuroblastoma cells [9–13]. We therefore investigated the molecular mechanisms of BetA-induced apoptosis in neuroblastoma cells.

MATERIALS AND METHODS

Drugs

BetA (Sigma, Deisenhofen, Germany) was provided as a pure substance and was dissolved in dimethylsulfoxide.

Cell Culture

Cells (SHEP, IMR-32, Kelly, LAN-5 neuroblastoma cell lines; Daoy, D283 Med, D341 Med, MHH1, MHH3,

MHH4, MEB1 medulloblastoma cell lines; A172, U118MG, U138MG, U251MG, U343, U373, SK14, SK17, SK19, SK22, SK37, SK49, SK51, SK55, SK60 glioblastoma cell lines; A17/95 and TC83 Ewing sarcoma cell lines; MCF-7 breast carcinoma, HT-29 colon carcinoma, KTCTL-26 renal cell carcinoma, H-146 small-cell lung carcinoma cells) were maintained in monolayer culture as previously described [4]. Preparation of tumor samples of 4 primary neuroblastoma stages IV or IVS, 4 primary medulloblastoma, and 24 primary glioblastoma was performed as previously described [4,10,12].

Determination of Apoptosis

Quantification of DNA fragmentation was performed by FACS analysis of propidium iodide-stained nuclei as previously described [4] using CELLQuest software (Becton Dickinson, Heidelberg, Germany).

Abbreviations: AIF, apoptosis inducing factor; BetA, betulinic acid; DiOC₆(3), 3,3'-dihexyloxycarbocyanide iodide; FACS, fluorescence-activated cell-sorting; PARP, poly(ADP-ribose) polymerase; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

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Western Blot Analysis

Immunodetection of caspase-8, caspase-3, PARP, and cytochrome *c* protein was performed by Western blot analysis as previously described [7].

Cell-Free System of Apoptosis

Preparation of mitochondria, cytosolic extracts, and nuclei was performed as previously described and was analyzed in a cell-free system of apoptosis as previously described [7].

Determination of Mitochondrial Membrane Potential

Mitochondrial membrane potential was determined as previously described using the fluorochrome DiOC₆(3) (Molecular Probes, Inc., Eugene, OR) [7].

RESULTS

We initially screened the cytotoxic effect of BetA on a panel of human tumor cell lines. BetA efficiently triggered apoptosis in neuroectodermal tumor cells including neuroblastoma, medulloblastoma, glioblastoma, and Ewing sarcoma cells [10]. Moreover, BetA triggered apoptosis in neuroblastoma cell variants that were resistant to anti-CD95 or doxorubicin, suggesting that BetA may bypass some forms of resistance [10]. Most importantly, BetA exhibited significant antitumor activity *ex vivo* on tumor cells derived from patients with neuroblastoma, medulloblastoma, or glioblastoma [10,12], indicating that BetA may be a promising new cytotoxic drug for the treatment of neuroectodermal tumors *in vivo*.

The mechanism of BetA-induced apoptosis differed from the mechanism of action previously identified for conventional cytotoxic drugs, e.g. doxorubicin. In contrast to many conventional cytotoxic drugs, BetA did not induce activation of the CD95 system or accumulation of wild-type p53 protein [10]. Treatment of neuroblastoma cells with BetA resulted in loss of the mitochondrial membrane potential, which was inhibited by the mitochondrion-specific inhibitor bongkreikic acid or in cells overexpressing Bcl-2 or Bcl-X_L [10]. Moreover, treatment with BetA induced cleavage of the receptor-proximal caspase-8, the effector caspase-3, and PARP [10]. Cleavage of caspase-8 occurred after the onset of loss of the mitochondrial membrane potential and was blocked by overexpression of Bcl-2 or Bcl-X_L [10], suggesting that caspase-8 was cleaved downstream of mitochondria. Cell-sorting experiments revealed that cleavage of caspases was confined to cells that had lost their mitochondrial transmembrane potential [10], providing further evidence that activation of caspases including caspase-8 occurred downstream of mitochondria. On treatment with BetA apoptogenic proteins such as cytochrome *c* or AIF were released from mitochondria into

the cytosol and might be involved in activation of caspases [7]. When tested on isolated mitochondria in a cell-free system, BetA directly triggered loss of the mitochondrial membrane potential independently of a zVAD.fmk-inhibitable caspase [11]. Moreover, mitochondria undergoing BetA-triggered permeability transition mediated activation of caspase-8, caspase-3, and PARP [12]. Soluble factors such as cytochrome *c* or AIF released from BetA-treated mitochondria were sufficient for activation of caspases and nuclear fragmentation [11]. Furthermore, addition of cytochrome *c* to cytosolic extracts induced cleavage of caspase-3, but not of caspase-8 [11]. However, supernatants of BetA-treated mitochondria or purified AIF activated both caspase-8 and -3 in cytosolic extracts and sufficed to activate recombinant caspase-8, suggesting that AIF but not cytochrome *c* may mediate cleavage of caspase-8 [11].

DISCUSSION

BetA, a pentacyclic triterpene derived from white birch trees, has recently been described as a novel cytotoxic compound against melanoma and neuroblastoma cells [9–13]. We identified BetA as a potent antitumor agent for the majority of solid tumors of childhood such as neuroblastoma, malignant brain tumors, and Ewing sarcoma and characterized the molecular mechanisms of BetA-induced apoptosis [10–12]. BetA exerted a cytotoxic effect on all neuroblastoma, medulloblastoma, glioblastoma, and Ewing sarcoma cell lines tested. BetA was also active against most primary tumor samples of patients with neuroblastoma, medulloblastoma, or glioblastoma, suggesting that BetA may exert antitumor activity *in vivo*.

Triggering of apoptosis by anticancer drugs involves activation of different signaling pathways including activation of death receptor systems, perturbation of mitochondrial function, and proteolytic processing of caspases [2]. Thus, the cell death pathway may be entered at different sites. BetA represents one class of anticancer agents that may act by directly targeting mitochondria. Using a cell-free system, we found that BetA directly triggered permeability transition in isolated mitochondria, and induction of permeability transition appears to be the initial event in BetA-triggered apoptosis. Mitochondrial perturbations on treatment with BetA resulted in the release of cytochrome *c* or AIF from mitochondria into the cytosol, where they induced activation of caspases and nuclear fragmentation leading to cell death. Overexpression of the antiapoptotic proteins Bcl-2 or Bcl-X_L, which inhibited mitochondrial perturbations and cytochrome *c* release from mitochondria, also inhibited activation of caspases and apoptosis. Because BetA triggered apoptosis in drug- and CD95-resistant neuroblastoma cells, direct activation of mitochondrial perme-

ability transition by cytotoxic drugs such as BetA may be sufficient for induction of apoptosis in cancer cells and may bypass some forms of tumor-associated mechanisms of chemoresistance.

CONCLUSION

BetA may be a promising new cytotoxic drug in the treatment of neuroectodermal tumors including neuroblastoma that clearly warrants further preclinical and clinical evaluation.

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