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Original article

Acetylenic derivative of betulin induces apoptosis in endometrial adenocarcinoma cell line

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ABSTRACT

Since betulin (Bet) and its acetylenic derivative, 28-O-propynoylbetulin (proBet) were shown to induce apoptosis in several cancer cell lines, we studied the mechanism of this process in human endometrial adenocarcinoma cells (EA). Previous studies suggested that this group of compounds affect prolidase activity (proline releasing enzyme from imidodipeptides) and collagen biosynthesis (proline utilizing process) providing substrate (proline) for proline oxidase (POX) dependent apoptosis. Here we provide evidence that Bet and proBet exhibit prolidase-inducing activity in EA cell line. However, in contrast to Bet, proBet inhibited collagen biosynthesis, increased intracellular proline concentration and induced apoptosis in EA cells, as detected by caspase-3, and -9 expressions and annexin V staining. Although POX expression was not affected by both compounds, the process of apoptosis was accompanied by increase in cytoplasmic level of proline. The mechanism for proBet-induced prolidase activity was found at the level of β1 integrin signaling. The inhibition of collagen biosynthesis was due to up-regulation of NF-κB p65, an inhibitor of collagen type I gene transcription. Although Bet and proBet induced expression of pro-apoptotic p53 in EA cells, the effect of proBet on the processes was much stronger. In contrast to proBet, Bet strongly induced expression of pro-survival factors, HIF-1α and VEGF. The data suggest that massive production of proline by proBet-dependent activation of prolidase and inhibition of proline utilization for collagen biosynthesis may represent mechanism for POX-dependent apoptosis in EA cells.

1. Introduction

In view of the fact that betulin and betulinic acid are considered as an inducers of apoptosis in cancer cells [1–4], it seems that this group of compounds may have anti-cancer potential. However, the detailed mechanism of apoptosis has not been reported yet. Previously we showed that betulinic acid evoked inhibition of collagen biosynthesis in human endometrial adenocarcinoma cells [2]. Inhibition of this process may contribute to increase in cytoplasmic level of free proline, providing substrate for proline oxidase (POX) that generates reactive oxygen species (ROS) inducing apoptosis [5].

We provided hypothesis that compounds of dual action: i/inhibiting collagen biosynthesis (the most effective proline utilizing process) and ii/inducing prolidase activity (proline releasing enzyme from imidodipeptides) may provide substrate (proline) for POX-dependent apoptosis.

Proline oxidase (POX) known also as proline dehydrogenase (PRODH, GenBank™ NM_016335) is flavin-dependent enzyme associated with the inner mitochondrial membrane [6,7]. It converts proline into Δ1-pyrroline-5-carboxylate (P5C). During this process electrons are transported to electron transport chain producing ATP for survival or they directly reduce oxygen, producing reactive oxygen species (ROS) that may induce apoptosis [6,8–10]. Therefore, POX may play dual role, evoking both tumor growth inhibiting or growth supporting activity. In the presence of proline, overexpression of POX causes cytochrome c release from mitochondria to cytosol and activation of caspase-9 and caspase-3 [10]. It seems that proline availability may represent the key factor in switching into apoptosis. Increase of cytoplasmic proline concentration could be a result of induction of prolidase activity and inhibition of collagen biosynthesis.

Prolidase (E.C.3.4.13.9) is a cytosolic exopeptidase of special interest since the enzyme activity may contribute to up-regulation not only cytoplasmic proline level but also transcriptional activity of HIF-1α and expression of VEGF [11,12]. The enzyme cleaves imidodipeptides with C-terminal proline or hydroxyproline, e.g. glycyl-proline

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[13]. The imidodipeptides are derived from intracellular degradation of collagen and other proline-containing proteins [14,15]. The enzyme recycles proline from imidodipeptides for collagen re-synthesis [16] and cell growth [17]. Prolidase activity is regulated by β1 integrin signaling [18]. Integrin receptors are heterodimers composed of α and β subunits. Integrin activation by ligands (e.g. collagen) result in activation of cytosolic β subunit associated signaling [19,20]. Non-receptor focal adhesion kinase (FAK) is the major transducer of signal generated by integrins. FAK is autophosphorylated at Tyr397 residue which is critical for subsequent phosphorylation of some tyrosine residues in FAK molecule [20,21]. Phosphotyrosine moiety serves as a site for association with signalling proteins such as growth factor receptor bound protein 2 (Grb2). Son of sevenless protein (Sos) associated with Grb-2 activates Ras and thereby triggers mitogen activated protein kinase cascade leading to phosphorylation of ERK1 and ERK2. Another protein activated by FAK is phosphoinositide 3-kinase involved in phosphorylation-dependent activation of Akt [19,22,23]. Activation of ERK 1/2 and Akt results in induction of transcription factors inducing gene expression of several proteins involved in regulation of cell growth and differentiation [24].

Collagen contains about 30% of proline and hydroxyproline residues. It is the most abundant proline-containing protein in the body. The process of collagen biosynthesis is stimulated by signals generated by insulin-like growth factor-I receptor (IGF-IR). IGF-I receptor signaling involves mostly the same proteins and kinases as the β1 integrin transduction pathway, except for the participation of FAK and Src kinases [25]. The end point of this process is induction of some transcription factors that activate collagen gene expression. One of transcription factors that inhibit collagen gene transcription is NF-κB [26].

For the reason we studied several betulin derivatives [27] among which we found its acetylenic derivative 28-O-propynoylbetulin (proBet, betulin monoester with −CO−C≡CH as acyl group at C28 position side chain) exhibiting prolidase-inducing and collagen-inhibiting activity. Characterization of this compound in respect to its effect on apoptosis and signaling mechanism in human endometrial adenocarcinoma cells is the aim of this study.

In this report we suggest that acetylenic derivative of betulin (proBet) up-regulates cytoplasmic proline level by activation of prolidase and inhibition of proline utilization for collagen biosynthesis, providing mechanism for POX-dependent apoptosis in EA cells.

2. Materials and methods

Betulin with a purity of ≥98% was purchased from Sigma-Aldrich. 28-O-propynoylbetulin was synthesized via esterification of the C-28 hydroxyl group of betulin with propynoic acid and characterized by standard spectroscopic methods. The ^{1}H NMR, ^{13}C NMR, IR and MS (EI) data of the compound were reported by Boryczka et al. [27]. Cisplatin, 5-fluorouracil, horseradish peroxidase conjugated anti-mouse IgG (A4416), anti-rabbit IgG (A9169) and anti-goat IgG (A5420) antibodies, bacterial collagenase, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), glycyl-L-proline, monoclonal (mouse) anti-pERK1/2 (M8159) and polyclonal (rabbit) anti-IκB-α (I0505) and anti-β-actin (A2066) antibodies, were provided by Sigma Corp., USA., as were most other chemicals and buffers used. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) used in cell culture were products of Gibco, USA. Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc., USA. Nitrocellulose membrane (0.2 μm), sodium dodecylsulphate (SDS), polyacrylamide, molecular weight standards and Coomassie Briliant Blue R-250 were received from Bio-Rad Laboratories, USA. L-5[^{3}H] proline (28 Ci/mmol) was purchased from Amersham, UK. Monoclonal (mouse) anti-p53 (sc-126), anti-POX (sc-376401) and anti-VEGF (sc-7269) antibodies, polyclonal (rabbit) anti-IGF-IR (sc-712), anti-NFκB p65 (sc-372), anti-FAK (sc-557), anti-Sos (sc-256), antibodies were the products of Santa Cruz Biotechnology Inc., USA. Monoclonal (rabbit) anti p-FAK

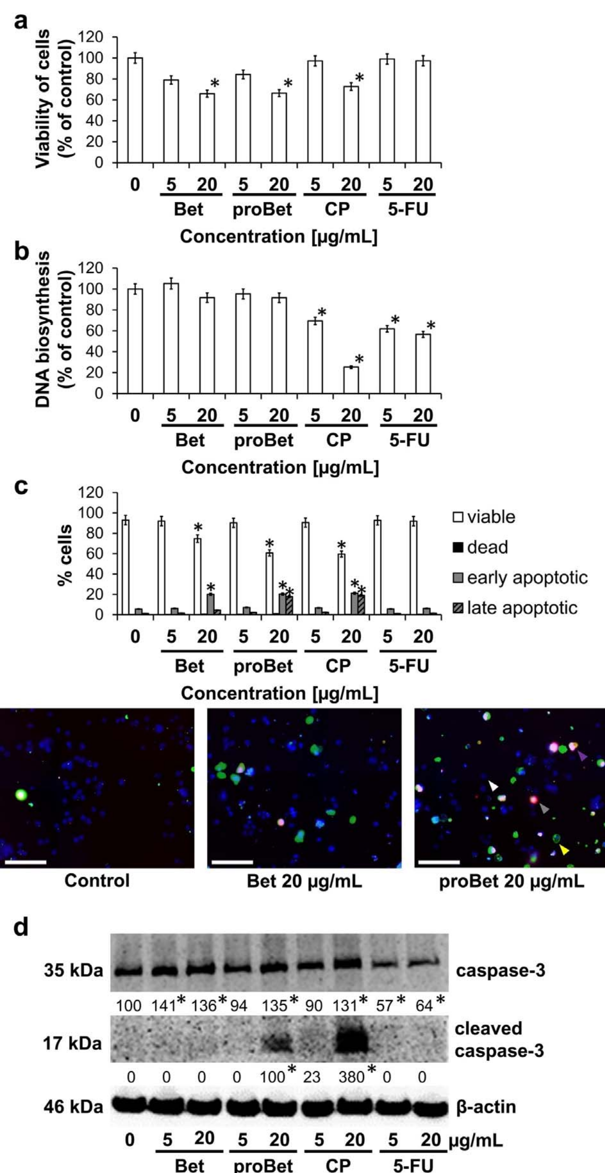

Fig 1. Viability (a), DNA biosynthesis (b), annexin V-FITC and propidium iodide staining (c) in human endometrial adenocarcinoma (EA) cells incubated for 24 h with different concentrations of betulin (Bet), 28-O-propynoylbetulin (proBet), cisplatin (CP) and 5-fluorouracil (5-FU). Representative merged photographs for annexin V (green), Hoechst 33342 (blue) and propidium iodide (red) stained cells are shown (c). Viable cell is indicated by white triangle, early apoptotic by yellow, late apoptotic by violet and dead by grey. Scale bar 50 μm. The results (a–c) present the mean values from 6 assays ± S.D. *P < 0.01. Western blot analysis for caspase-3 and cleaved caspase-3 (d) in EA cells incubated for 24 h with different concentrations of Bet, proBet, CP and 5-FU. The mean values of six pooled cell homogenate extracts from six separate experiments are presented. The intensity of the bands was quantified by densitometry analysis. Densitometry was done with BioSpectrum Imaging System and presented as an arbitrary units. Numbers indicate results of densitometry analysis of proteins with normalization to β-actin levels. Significant (P < 0.05) changes in band intensities compared with control are indicated by *. The same amount of supernatant protein (20 μg) was run in each lane. The expression of β-actin served as a control for protein loading.

(Tyr397) (700255) was the product of Invitrogen. Polyclonal (rabbit) anti-caspase-3 (9662) and anti-ERK1/2 (9102), monoclonal (rabbit) anti-pAkt (Thr308) (2965), anti-Akt (4685), anti-pAMPKα (Thr172) (4188) and monoclonal (mouse) anti-caspase-9 (9508) antibodies were the products of Cell Signaling Inc., USA. Monoclonal (mouse) anti-β1 integrin (610468), anti-Grb-2 (610112), anti-AMPKβ (610802) and anti-HIF-1α (610959) antibodies were obtained from Becton Dickinson Co., USA. Polyclonal (rabbit) anti-prolidase (ab86507) antibody was

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