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Pentoxifylline augments TRAIL/Apo2L mediated apoptosis in cutaneous T cell lymphoma (HuT-78 and MyLa) by modulating the expression of antiapoptotic proteins and death receptors

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) is a promising anticancer agent but cutaneous T lymphoma cells (CTCL) are less sensitive to TRAIL-induced apoptosis. Here, we report that pentoxifylline (PTX), a phosphodiesterase inhibitor, augments TRAIL-mediated apoptosis in HuT-78 and MyLa cells through modulating extrinsic death receptors and intrinsic mitochondria dependent pathways. Our results clearly show that PTX augments TRAIL-mediated activation of caspase-8 and induces cleavage of Bid, although PTX alone cannot activate caspase-8. This is followed by cytochrome *c* release and subsequent, activation of caspase-9 and caspase-3 and cleavage of poly (ADP ribose) polymerase (PARP). Combined treatment downregulates the expression of various antiapoptotic proteins including c-FLIP, Bcl-xl, cIAP-1, cIAP-2 and XIAP. PTX induces the expression of death receptors DR4 and DR5 on cell surface of both the cell types where c-Jun NH2-terminal kinase (JNK) pathway plays an important role. Moreover, combined silencing of DR4 and DR5 by small interfering RNA abrogates the ability of PTX to induce TRAIL-mediated apoptosis. Thus, this is the first demonstration that PTX can potentiate TRAIL-mediated apoptosis through downregulation of cell survival gene products and upregulation of death receptors.

1. Introduction

Cutaneous T cell lymphomas (CTCL) are lymphoproliferative disorders of the skin. The two most common forms of CTCL are Mycosis fungoides (MF) and Sézary syndrome (SS) as its leukemic form, together they account for majority of cutaneous lymphoma [1]. CTCL cells show defects in their death inducing signaling complex due to altered expression of death receptors which make them less sensitive toward TNF superfamily molecules [2,3]. It has been reported that circulating CD4+ cells from SS patients are resistant to soluble TRAIL [3].

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) has recently emerged as a novel anticancer agent based on its ability to induce apoptosis in tumor cells, without showing toxicity to normal cells [4]. TRAIL binds to four membrane bound receptors DR4/TRAIL-R1, DR5/TRAIL-R2, DcR1/TRAIL-R3, DcR2/TRAIL-R4 and a soluble receptor osteoprotegerin (OPG). TRAIL transduces its signal only through DR4 and DR5 which contain cytoplasmic region called the death domain. DcR1 and DcR2 do not have functional cytoplasmic death domain and functions as blocker of TRAIL-induced apoptosis [5]. Binding of TRAIL to DR4 and DR5 leads to the formation of death inducing signaling complex (DISC) with the binding of caspase-8 which is then activated. Activation of caspase-8 ultimately activates caspase-3 leading to cell death. Alternatively, TRAIL can also activate caspase-3 through caspase-9 pathway where mitochondria are involved [6]. The presence of intracellular apoptosis inhibitory substances (Bcl-xl, c-FLIP, cIAP etc.) have been shown to play an important role in TRAIL resistance in different cancer cells [7]. Numerous studies demonstrated that combination of TRAIL with chemotherapeutic drugs or ionizing radiation can induce synergistic tumor cell apoptosis by upregulating DR4 and/or DR5 or by inhibiting intracellular cell survival proteins [5,8]. Earlier, we reported that PTX, a methylxanthine derivative induces FasL mediated killing in human sézary CTCL cell line, HuT-78 by upregulating the expression of Fas receptors on their cell surface [9].

Cyclic nucleotide phosphodiesterase inhibitor, Pentoxifylline (PTX), has been shown to beneficially influence a large number of inflammatory skin diseases and now being clinically used as peripheric vasodilator [10,11]. PTX has been shown to enhance

Abbreviations: CTCL, cutaneous T cell lymphoma; MF, mycosis fungoides; SS, sézary syndrome; PTX, pentoxifylline; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; c-FLIP, cellular FLICE like inhibitory protein; cIAP, cellular inhibitor of apoptosis protein; XIAP, X-linked inhibitor of apoptosis; JNK/MAPK, c-Jun NH2-terminal kinase/mitogen-activated protein kinase; DR4, death receptor 4; DR5, death receptor 5; DcR, decoy receptor.

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tumor sensitivity of many chemotherapeutic agents as well as sensitize tumor cells to ionizing irradiation [12–15]. Recently, PTX in combination with Vitamin E have been found beneficial for radiation induced fibrosis and survival in patients with non-small cell lung cancer [10]. Presently, only limited compounds are approved by Food and Drug Administration (FDA) for the treatment of CTCL patients and therapies available are usually palliative [16–18]. Therefore, alternative or complementary therapies, especially for advanced MF/SS, are urgently required [19]. Earlier, gene expression profiling of aggressive CTCL patients showed an overexpression of TRAIL, but the expression of TRAIL receptors has not been correlated with TRAIL sensitivity in SS tumor cells [3].

Present work was undertaken to see the effect of PTX on TRAIL-induced apoptosis in CTCL cell lines (HuT-78 and MyLa). Here, we report for the first time, that PTX augments TRAIL-mediated caspase-8 activation and potently induces TRAIL-mediated apoptosis in both the cell lines by downregulating different antiapoptotic proteins and upregulating DR4 and DR5.

2. Materials and methods

2.1. Chemicals and antibodies

Human CTCL cell lines, HuT-78 was obtained from National Centre for Cell Science (Pune, India) and MyLa was generously provided by Dr. Emmanuel Contassot (Department of Dermatology, Zürich University Hospital, Zürich, Switzerland). Both the cell lines were maintained in RPMI-1640 medium, containing 10% heat inactivated Fetal Bovine Serum (GIBCO, Grand Island, NY) at 37 °C and in the presence of 5% CO_2. Human recombinant TRAIL was purchased from Millipore/Chemicon (Billerica, MA). JNK inhibitor, SP600125, caspase inhibitors, Z-VAD-FMK and Z-IETD-FMK were purchased from Calbiochem (La Jolla, CA). Antibodies against c-FLIP, t-Bid, ERK, p-ERK, p38, p-p38, DR4 and DR5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PI/RNase staining buffer, annexin V-FITC apoptosis detection kit, APO-BRDU™ kit and antibodies against cytochrome *c*, PARP, Bcl-xl, Apaf-1, cIAP-1, cIAP-2, XIAP, JNK1/JNK2, JNK/SAPK (pT183/pY185), PE-labeled IgG1 isotype control were purchased from BD PharMingen (San Diego, CA). PE-labeled TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1 and TRAIL-R4/DcR2 antibodies for flowcytometry and neutralizing human anti-TRAIL antibody (RIK-2) were purchased from e-Biosciences (San Diego, CA). Quick-Load™ 2-Log DNA Ladder was purchased from New England Biolabs (Ipswich, MA). Pentoxifylline (PTX), acridine orange (AO), ethidium bromide (EB), propidium iodide (PI), p-formaldehyde, 4,6-diamidino-2-phenyl-indole (DAPI), anti-actin antibody, anti-mouse HRP, anti-rabbit HRP and anti-goat HRP antibodies were purchased from Sigma–Aldrich (St. Louis, MO). All media and reagents used were endotoxin free.

2.2. PI exclusion assay for cell viability

Cell viability was determined by PI exclusion assay [9]. Briefly, 2×10^5 HuT-78 and MyLa cells each were treated with different concentrations of PTX (0, 1.5 and 3 mg/mL) or TRAIL (0, 25, 50 and 100 ng/mL) either alone or together for 24 h. After treatment, cells were harvested, washed with phosphate buffered saline (PBS), and resuspended in PBS containing 1 μg/mL of PI. The level of PI incorporation was quantified by flow cytometry on a FACSCalibur (Becton Dickinson).

2.3. Fluorescence morphological examination

Cell morphology was investigated by staining cells with a combination of fluorescent DNA binding dyes AO/EB. Briefly, 2.5×10^5 HuT-78 cells were treated with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together for 24 h. The solution containing each dye at 1 μg/mL in PBS was mixed 1:1 with cell suspension. Cell viability was determined under Zeiss fluorescence microscope using a 40× objective by counting live (green) and dead (red) cells [9].

2.4. Quantification of apoptosis by flow cytometry

2.4.1. Analysis of hypoploidy

1×10^5 HuT-78 cells were treated with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together for 24 and 48 h. After incubation, cells were harvested, fixed in 70% ethanol and stained with PI/RNase staining buffer (5 μg/mL PI, 200 μg/mL RNase) as described [20]. The percentage of apoptotic cells was determined by measuring the fraction of nuclei with a sub-diploid DNA content using Becton Dickinson FACSCalibur with Cell Quest software (Becton Dickinson).

2.4.2. Annexin V staining

HuT-78 cells were treated with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together for 24 and 48 h. Cells were harvested, washed and stained with 5 μL of fluorescein isothiocyanate (FITC)-conjugated annexin V using Annexin V staining kit (BD Pharmingen) according to manufacturer's protocol. Stained cells were immediately analyzed with a Becton Dickinson FACSCalibur and further analyzed using CellQuest software (Becton Dickinson).

2.4.3. Terminal deoxynucleotidyltransferase dUTP nick end labelling (TUNEL)

To measure the DNA strand breaks during apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay was performed using APO-BRDU™ kit (BD Pharmingen) according to manufacturer's protocol. In brief, 5×10^5 HuT-78 and MyLa cells each were treated with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together for 24 and 48 h. Cells were harvested, washed with PBS and stained by incubating with reaction mixture. Stained cells were analyzed with flow cytometer (FACSCalibur, Becton Dickinson).

2.5. DAPI staining

For analysis of nuclear morphology, cells were harvested after treatment, washed twice with PBS and fixed in 1% p-formaldehyde. After fixation, staining was carried out by incubating cells with 10 μL of 4 μg/mL DAPI for 10 min in dark. Later samples were visualized under Zeiss fluorescence microscope [21].

2.6. DNA fragmentation analysis

Cells were harvested after treatment and DNA fragmentation was assayed according to the method described by Herrmann et al. [22].

2.7. Measurement of caspase activity

2.7.1. Caspase-8 and caspase-3 activity assay

Caspase-8 (Sigma–Aldrich, St Louis, MO) and caspase-3 (Promega, Madison, WI) activities were determined according to manufacturer's protocol. Cell lysates were prepared after treatment with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together for 24 h. After treatment cells were harvested and assayed for caspase-8 and caspase-3 activity using colorimetric substrates. The specificity of caspase-8 and 3 activities were checked by using their specific inhibitors Z-IETD-FMK and Z-VAD-FMK respectively.