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Pouterin, a novel potential cytotoxic lectin-like protein with apoptosis-inducing activity in tumorigenic mammalian cells ☆

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ABSTRACT

In this study, the cytotoxicity of pouterin in tumorigenic and non-tumorigenic mammalian cell lines was investigated. We found that HeLa, Hep-2 and HT-29 tumor cells were highly sensitive to pouterin cytotoxicity in a dose-dependent manner, whereas non-tumorigenic Vero cells and human lymphocytes were relatively resistant to the protein. Among the tumor cell lines, HeLa cells showed the highest susceptibility to pouterin cytotoxicity, exhibiting a time-dependent increase in LDH leakage and an IC_{50} value of 5 μg/mL. Morphological alterations such as rounding, cell shrinkage and chromatin condensation, consistent with apoptotic cell death were observed. Apoptosis induction was demonstrated by DNA fragmentation as detected by terminal dUTP nick-end labeling (TUNEL). Furthermore, HeLa cells incubated with pouterin showed disruption of the actin cytoskeleton. Western blot analysis revealed that pouterin caused increased expression of p21, thus indicating cell cycle arrest. Subsequent studies provided evidence that apoptosis may be partially explained in the activation of the tumor necrosis factor receptor 1 (TNFR1) signaling. Interestingly, a time-dependent decrease of the expression of p65 nuclear factor kappa B (NFκB) subunit, concomitant with a downregulation of the inhibitor of apoptosis protein 1 (IAP1) was observed, suggesting that TNFR-mediated apoptosis is the predominant pathway induced by pouterin in HeLa cells.

1. Introduction

Apoptosis is a complex process whereby intrinsic and extrinsic stimuli activate a molecular program to implement a specific series of events that culminate in cell death. Circumstantial evidence of apoptosis induction, due to effects on cell cycle or DNA integrity and the presence of several oncogenes that promote cell cycle progression, also inducing apoptosis, has implied the existence of a direct relationship between these two antagonistic processes (Büssing et al., 1998). Cells undergoing apoptosis exhibit specific morphological changes, which include membrane blebbing, cytoplasm and chromatin condensation, nuclear breakdown and formation of

☆ *Ethical statement*: We would like to inform that this manuscript did not utilize experiments with animals and humans. The study was realized with in vitro experiments.

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apoptotic bodies, which are eventually subjected to phagocytosis (Rao et al., 2005; Bortner and Cidlowski, 2007).

Cell death with apoptotic morphology can be triggered by several stimuli, including intracellular stress and receptor-mediated signaling. These signals mainly converge in the activation of intracellular caspases, a family of conserved proteases, which propagate death signaling by cleaving key cellular substrates such as enzymes involved in genome function, regulators of the cell cycle and structural proteins of the nucleus and cytoskeleton (Büssing et al., 1998; Vermeulen et al., 2005). Apoptosis can be initiated by the ligation of members on the tumor necrosis factor (TNF) receptor family, such ligands/receptors include Fas/Apo-1/CD95 and TNF receptor 1 (TNFR1). Ligand binding triggers receptor oligomerization, resulting in the recruitment of the adaptor protein TNFR1-associated death domain (TRADD), which serves as a platform for binding of Fas-associated death domain (FADD) and TNFR-associated factor 2 (TRAF2) to the receptor complex. The latter can then promote the activation of caspase 8, one of the initiator apoptotic proteases (Baker and Reddy, 1998; Wallach et al., 1999). However, through the binding of TRADD to the downstream transducer TRAF2 and the protein kinase nuclear factor kappa B (NFκB)-inducing kinase (NIK), TNFR1 also activates the transcription factor NFκB, which activates transcription of polypeptides such as inhibitor of apoptosis proteins (IAP1 and IAP2) that antagonize the death signals (Wang et al., 1998; Deveraux and Reed, 1999). Transcription factors such as NFκB have been implicated in both carcinogenesis and the development of drug resistance in cancer cell (Hanahan and Weinberg, 2000). While activation of NFκB may induce apoptosis in certain situations (Chuang et al., 2002), most reports suggest that NFκB mediates survival signals that counteract apoptosis (Escárcega et al., 2007). While intrinsically or constitutively activated NFκB may be critical in the development of drug resistance in cancer cells, transient, inducible activation of NFκB may be as important, but is not as well studied (Chuang et al., 2002). Much effort is currently invested in developing various inhibitors of this pathway and testing their efficacy in human cancer (Monks et al., 2004).

Studies have shown that lectins and proteins with lectin-like activity trigger various biological effects. Some lectins such as wheat germ agglutinin (WGA), concanavalin A (Con A), ricin, mistletoe, phytohemagglutinin (PHA), and the trypsin inhibitor from *Peltophorum dubium* seeds (PDTI) with lectin-like properties can kill normal and malignant cells at relatively low concentrations (Kim et al., 1993; Büssing, 1996; Troncoso et al., 2003). Some lectins have recently been shown to induce apoptosis, and this could explain their cytotoxicity (Hajtó et al., 2003; Ohba et al., 2004; Rao et al., 2005). Recently, we purified and characterized a protein from the seeds of *Pouteria torta* (Sapotaceae) with lectin-like activity (Boleti et al., 2007). This protein (pouterin) showed one protein band in SDS-PAGE (Mr 14 kDa) and agglutinated human and animal erythrocytes. Furthermore, the pouterin protein has fungitoxic and insecticidal properties, but its cytotoxicity in mammalian cells has not been studied. The present study was designed to investigate the cytotoxic activity of pouterin in various mammalian cell lines and its ability to induce apoptosis. Our results indicated that pouterin is a highly cytotoxic protein to tumor cells. Among the cell lines tested, a higher susceptibility to pouterin toxicity was observed for HeLa cells. In addition, the results also suggested that the underlying cytotoxic mechanism of pouterin was mediated by apoptosis through upregulation of the TNFR1 and down-modulation of NFκB expression. Additionally, pouterin induced disruption of the actin cytoskeleton, which may be coupled with the apoptotic cell death.

2. Materials and methods

2.1. Reagents

Antibodies against receptor-associated factor 2 (TRAF2), Bax, tumor necrosis factor receptor 1 (TNFR1), TNF receptor-associated death domain (TRADD) and inhibitory apoptosis protein type 1 (IAP1) and p21 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibody against NFκB p65, and anti-rabbit and anti-mouse peroxidase-conjugated antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Fluorescein isothiocyanate-labeled phalloidin was from Sigma Chemical Co. (St. Louis, MO, USA), and the *in situ* cell death detection kit (ApopTag) and the colorimetric cytotoxicity detection kit (LDH) were from Roche Applied Science (Mannheim, Germany).

2.2. Pouterin purification

Pouterin was prepared according to Boleti et al. (2007). Dehulled *P. torta* seeds were finely ground and extracted with 150 mM NaCl (1:5 meal to buffer ratio) for 24 h at 4 °C and then centrifuged at 10,000*g* for 30 min at the same temperature. The clear supernatant (crude extract) was used to determine the protein content and hemagglutinating activity. The extract was diluted in 100 mM phosphate buffer, pH 7.6, containing 100 mM NaCl and applied to a Sephacryl S-100 column (2.5 cm × 40 cm) equilibrated with the same solution. The fraction was applied to a DEAE-Sepharose column (2 cm × 20 cm) equilibrated with 50 mM Tris–HCl buffer, pH 8.0, and eluted with a linear gradient of NaCl (0–1.0 M). The lectin-like protein fraction was dissolved in 0.1% (v/v) trifluoroacetic acid (solvent A) and clarified by centrifugation. The resulting supernatant was applied to a μ-Bondapack C_{18} column (0.78 cm × 30 cm, Waters 991-PDA system) and the proteins were eluted with a linear gradient (0–100%, v/v) of acetonitrile (solvent B, 66% acetonitrile in 0.1% trifluoroacetic acid) at a flow rate of 2 mL/min. The hemagglutinating activity of pouterin was monitored during purification and the purity of pouterin was checked by SDS-PAGE 6 (Laemmli, 1970). The purified protein was extensively dialyzed and lyophilized prior to storage at −20 °C.