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Anticancer mechanisms of temporin-1CEa, an amphipathic α-helical antimicrobial peptide, in Bcap-37 human breast cancer cells

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ABSTRACT

Aims: Temporin-1CEa, a 17-residue antimicrobial peptide, is known to exert broad-spectrum anticancer activity that acts preferentially on cancer cells instead of normal cells. However, the mechanism of cancer cell death induced by temporin-1CEa is weakly understood.
Main methods: Here, we investigated the cytotoxic and membrane-disrupting effects of temporin-1CEa on human breast cancer cell line Bcap-37, using MTT assay, electronic microscope observation, fluorescence imaging and flow cytometry analysis.
Key findings: The MTT assay indicated that one-hour temporin-1CEa treatment led to rapid cell death in either caspase-dependent or -independent manner. The electronic microscope observation suggested that temporin-1CEa exposure resulted in profound morphological changes in Bcap-37 cells. The fluorescence imaging and flow cytometry analysis demonstrated that temporin-1CEa exhibited membrane-disrupting property characterized by induction of cell-surface phosphatidylserine exposure, elevation of plasma membrane permeability, and rapid transmembrane potential depolarization. Moreover, temporin-1CEa might also induce rapid cell death through mitochondria-involved mechanisms, including rapid intracellular Ca^{2+} leakage, collapse of mitochondrial membrane potential ($\Delta\varphi m$) and over-generation of reactive oxygen species (ROS).
Significance: In summary, the present study indicates that temporin-1CEa triggers a rapid cytotoxicity in Bcap-37 cells through membrane-destruction and intracellular mechanisms involving mitochondria. These intracellular mechanisms and direct membrane-destruction effect were evaluated helping to understand the detail action of antimicrobial peptides in mammalian cancer cells.

Introduction

A major limitation inherent to most conventional anticancer chemotherapeutic agents is their lack of tumor selectivity and therefore the deleterious side effects. Moreover, cancer cells can develop resistance to conventional chemotherapy agents by cellular changes through multimechanisms (Gatti and Zunino, 2005). These limitations associated with conventional chemotherapy have stimulated the search for new classes of anticancer drugs with new modes of action.

Antimicrobial peptides (AMPs) recently have received attention as alternative chemotherapeutic agents that overcome the limitations of current drugs. AMPs have several advantages over currently used oncolytic therapeutics, such as selective cytotoxicity for cancer cells, ability to bypass the multidrug-resistance mechanism, and additive effects in combination therapy (Papo and Shai, 2005). AMPs are expressed in many diverse species and have an important function in the host innate immunity to microbial pathogens (McPhee and Hancock, 2005; Zasloff, 2002). In addition to antimicrobial activity, some certain kinds of synthetic AMPs or natural AMPs, including cecropin B, magainins, melittin, tachyplesin, BMAP-28 and lactoferrin, have recently been shown to exert exciting potentials as a new class of anticancer agents. These AMPs exert rapid and selective cytotoxicity against malignant cells but show relatively lower cytotoxicity against untransformed proliferating cells (Eliassen et al., 2003; Furlong et al., 2006; Mader et al., 2005; Risso et al., 1998; Simmaco et al., 1990; Wang et al., 2012), thereby suggesting that these peptides may be administered in vivo with minimal nonspecific toxicity. The cytotoxic effect of these AMPs on microorganisms and neoplastic cells is commonly believed to be a function of the cationic nature and secondary structure of these peptides. Due to the net positive charge and amphipathic structure of AMPs, the direct interaction of these peptides with plasma membrane is a distinct mechanism from those conventional chemotherapeutic agents that are currently used in the treatment of human malignancies, thus preventing the development of side-effects and multiple drug resistance (Leuschner and Hansel, 2004; Mader and Hoskin, 2006).

Temporin-like peptides were initially identified in methanol extracts of the skins of the Asian frog *Rana erythraea* and the European hybrid frog *Rana esculenta* (Simmaco et al., 1990). In 1996, 10 structurally

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related peptides, endowed with antimicrobial properties, were discovered (Simmaco et al., 1996). These peptides were isolated from skin secretions of mild electrically stimulated specimens of the European red frog *Rana temporaria* and were designated as temporins from A to L. New members were identified in skin secretions of other ranid frogs of both North American and Eurasian origin, thereby enlarging the temporin family to more than 100 different isoforms (Mangoni, 2006; Mangoni and Shai, 2009). Previous research have reported that two temporin-like peptides, temporin-1DRa and temporins-ALa, exert potent anticancer activities (Conlon et al., 2007; Lu et al., 2006). Recently, temporin-1CEa, one novel 17-residue (FVDLKKIANIINSIFGK) AMP isolated from the skin secretions of the Chinese brown frog (*Rana chensinensis*), has been shown to exhibit a rapid cytotoxicity against human breast cancer cell lines (Shang et al., 2009; Wang et al., 2012). Moreover, temporin-1CEa has a lower hemolytic effect on human erythrocytes and has no cytotoxicity against normal human umbilical vein smooth muscle cells at concentrations that induce cancer cell death (Wang et al., 2012).

The anticancer mechanisms of temporin-1CEa against two human breast cancer cell lines, MCF-7 and MDA-MB-231, have been explored in our previous study, in which temporin-1CEa induces significant membrane disruptions, Ca^{2+}-releasing and ROS over production (Wang et al., 2013). In the present study, to clarify the cell line specificity of anticancer activity of temproin-1CEa, the anticancer activity of temporin-1CEa was further evaluated using Bcap-37, an ERα negative human breast cancer cell line that first established in China. The influences of temporin-1CEa on cell membrane and possible intracellular mechanisms of temporin-1CEa-induced cancer cell death were also investigated and discussed.

Materials and methods

Cell culture

The human breast cancer cell line, Bcap-37, was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100-U/mL penicillin and 100-U/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2 (Wang et al., 2009).

Effects of caspase inhibitors on temporin-1CEa-induced Bcap-37 cell death

To clarify the possible involvement of capspase-related pathways in temporin-1CEa induced cancer cell death, caspase-3 inhibitor (Ac-DEVD-fmk), caspase-8 inhibitor (Ac-IETD-fmk), or caspase-9 inhibitor (Ac-LEHD-fmk) of 100 μM concentration were co-incubated with Bcap-37 cells for 60 min. Then cells were exposed to various concentrations of temporin-1CEa (20–40 μM) for 1 h. The cells' death and sensitivities' cell death to caspase inhibitors were evaluated using a 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Morphological analysis using electronic microscope

Some AMPs can target nonpolar lipid cell membranes resulting in the target cells death. Therefore, in the present study, morphological changes of Bcap-37 cells after 1 h peptide treatment were examined by scanning electronic microscopy (SEM, KYKY-1000B) and transmission electron microscope (TEM, JEM-200EX) using standard protocols.

Assessment of cell-surface phosphatidylserine exposure and plasma membrane integrity using FITC-annexin V and propidium iodide (PI) staining

To evaluate the effects of temporin-1CEa on cell-surface phosphatidylserine (PS) exposure and plasma membrane integrity, Bcap-37 cells were seeded in a 96-well plate and incubated with various concentrations of temporin-1CEa (20–40 μM) or were left untreated (control) for 60 min. After treatment with peptides, the cells were stained with FITC-annexin V and PI according to manufacturer's instructions (FITC-Annexin V Apoptosis Detection Kit, BD Biosciences, USA). The cell-surface phosphatidylserine (PS) exposure and plasma membrane integrity were analyzed using FACSCanto flow cytometer (BD Biosciences). Cells that are considered viable are FITC-annexin V and PI negative (lower-left quadrant, Q3); cells with membrane lipid asymmetry and PS exposure are FITC-annexin V positive (lower-right quadrant, Q4); and cells with interrupted membrane integrity are both FITC-annexin V and PI positive (upper-right quadrant, Q2).

Cell membrane permeability assay using calcein AM and ethidium homodimer (EthD-1) staining

The cell membrane integrity and permeability were determined using the LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Molecular Probes, Inc., USA), which is a two-color fluorescence assay with two probes that measure recognized parameters of cell viability, including intracellular esterase activity and plasma membrane integrity. An increased fluorescence intensity of EthD-1 or a decreased fluorescence intensity of calcein means enhanced membrane permeability and interrupted membrane integrity. Bcap-37 cells were seeded into 96-well plates at 5×10^4 cells/mL. After treatment with temporin-1CEa (20–40 μM) or were left untreated (control) for 60 min, the medium was removed, and 20 μL of dye containing 2 μM calcein AM and 4 μM EthD-1 was then added and incubated for 30 min in the dark. The fluorescence intensity was distinguished by FACS analysis with Ex485nm/Em530nm for calcein and Ex530nm/Em645nm for EthD-1.

FITC-labeled peptides uptake

To detect the possible dynamic processing of temporin1CEa through cell membranes, Bcap-37 cells were incubated with FITC-labeled temporin-1CEa (20–40 μM) for 5, 10 or 60 min. Myelin and other lipophilic areas on cell membrane were stained with the red-orange fluorescent tracker DiI (Molecular Probes, Inc., USA). The FITC-labeled peptides were traced and recorded at each time point using laser scanning confocal microscopy.

Transmembrane potential measurements

Since membrane depolarization has been implicated in the mode of action of AMPs, cell transmembrane potential depolarization was measured using the membrane potential sensitive dye, bis-(1,3-dibutylbarbituric acid) trimethin eoxonol [$DiBAC_{4(3)}$]. As previously described (Franco et al., 2006), after incubation with 2 μM $DiBAC_{4(3)}$ for 10 min at 37 °C, the cells were subjected to time scanning using a fluorescence spectrophotometer (Varioskan Flash, Thermo Scientific) with Ex488 nm/Em518 nm. When the fluorescence intensity was stable, the cells were treated with either temporin-1CEa or sterile-deionized water. Membrane depolarization was monitored by observing the changes in the intensity of fluorescence emission of the membrane potential dye $DiBAC_{4(3)}$.

Cytosolic calcium (Ca^{2+}) concentration determination

To detect cytosolic Ca^{2+} levels, the Ca^{2+}-specific fluorescent dye, Fluo3-AM, was loaded into Bcap-37 cells using a modified procedure adapted from the manufacturer (Beyotime, China). Briefly, cells were incubated for 30 min at 37 °C with 4 μM Fluo3-AM in Hank's buffered salt solution (HBSS) with or without 1.3 mM Ca^{2+}. The cells were then washed two times with fresh HBSS and incubated in HBSS at room temperature prior to detection. The Fluo3-AM green fluorescence (Ex488 nm/Em526 nm) was proportional to cytosolic Ca^{2+} levels. The changes of Fluo3-AM intensity in response to the peptides in cell populations were monitored using flow cytometry.