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Generation of a hematologic malignancy-selective membranolytic peptide from the antimicrobial core (RRWQWR) of bovine lactoferricin

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ABSTRACT

Cationic antimicrobial peptides such as bovine lactoferricin (LfcinB) constitute an important innate defense mechanism against many microbial pathogens. LfcinB also binds to and selectively kills human cancer cells via a mechanism that involves reactive oxygen species (ROS) generation and caspase activation. The antimicrobial core of LfcinB consists of only six amino acids (RRWQWR), referred to in this study as LfcinB6. Although free LfcinB6 is devoid of cytotoxic activity against cancer cells, we show here that adding a cell-penetrating hepta-arginine sequence via a glycine–glycine linker to LfcinB6 generates a peptide (MPLfcinB6) that is selectively cytotoxic for human T-leukemia and B-lymphoma cells. Flow cytometric analysis of propidium iodide and fluorescein isothiocyanate-dextran uptake by MPLfcinB6-treated cancer cells revealed extensive damage to the cell membrane, which was confirmed by scanning electron microscopy. MPLfcinB6-induced cytotoxicity was also associated with sequential ROS production and mitochondrial membrane permeabilization; however, neither ROS nor caspase activation caused by the loss of mitochondrial membrane integrity was essential for peptide-mediated cell death. We conclude that MPLfcinB6 selectively kills human T-leukemia and B-lymphoma cells by causing extensive and irreparable damage to the cell membrane.

Introduction

Cationic antimicrobial peptides typically consist of fewer than 50 amino acid residues and act as an important aspect of the innate immune system of most organisms (Hancock, 2001). These host defense peptides are predominantly composed of basic (positively charged) and hydrophobic amino acids that adopt an amphipathic secondary structure when interacting with biological membranes (Bulet et al., 2004). The positively charged amino acids facilitate peptide aggregation to negatively charged membranes, allowing the hydrophobic residues to integrate into the lipid bilayer, thereby anchoring the peptide to, and allowing the disruption of, biological membranes. In addition to antibacterial properties (Powers and Hancock, 2003), many cationic antimicrobial peptides exert antiviral (Chattopadhyay et al., 2006), antiparasitic (Aley et al., 1994), antifungal (Lopez-Garcia et al., 2005), immunomodulatory (Choi et al., 2012), and anticancer effects (Hoskin and Ramamoorthy, 2008). Cationic antimicrobial peptides that are cytotoxic for cancer cells act by either causing the target cells to lyse or by initiating apoptosis upon gaining access to, and damaging, cell organelles such as mitochondria. The selective killing of cancer cells by cationic antimicrobial peptides is thought to be largely due to differences between the cell membrane of cancer cells and normal cells in that the former carry a net negative charge on their outer membrane leaflets whereas the latter are neutral in charge (Dobrzynska et al., 2005 and Iwasaki et al., 2009).

Bovine lactoferricin (LfcinB) is a lactoferrin-derived cationic antimicrobial peptide that kills a variety of cancer cells without harming normal, healthy cells (Mader et al., 2005). Interestingly, the mechanism of LfcinB-induced cytotoxicity differs between cancer cell types, i.e., LfcinB-treated T-leukemia cells die by caspase- and reactive oxygen species (ROS)-dependent apoptosis (Mader et al., 2005, 2007) whereas LfcinB-treated fibrosarcoma and neuroblastoma cells die by a cytolytic mechanism (Eliassen et al., 2002, 2006). The antimicrobial core of LfcinB resides in a six amino acid sequence (RRWQWR) that corresponds to amino acid residues 4–9 of full-length LfcinB (Schibli et al., 1999). In an earlier study, we evaluated the anticancer potential of the

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antimicrobial core of LfcinB (referred to as LfcinB6) on Jurkat T-leukemia cells and determined that LfcinB6 by itself is not cytotoxic whereas encapsulation of LfcinB6 within a fusogenic liposome that mediates intracellular delivery results in potent cytotoxic activity that involves the action of caspases and cathepsin B but does not require ROS generation (Richardson et al., 2009). However, intracellular delivery of LfcinB6 via fusogenic liposomes has a major limitation in that the peptide-containing liposomes also fuse with, and kill, normal cells.

In this study, we show that a cell-penetrating peptide domain is able to serve as an alternative mechanism for targeting LfcinB6 to cancer cells. Cell-penetrating peptides traverse the cell membrane by a poorly understood mechanism, resulting in delivery of covalently bound cargo such as chemotherapeutic drugs and solid nanoparticles to the cytosolic compartment of the target cell (Choi et al., 2011). A consecutive sequence of at least seven basic amino acids (arginine outperforms lysine and histidine) is required for effective transport of cargo across the cell membrane (Mitchell et al., 2000). In this regard, the selective anticancer activity of an artificial polycationic KLA peptide is substantially enhanced when the peptide is conjugated to a cell-penetrating hepta-arginine (R7) domain (Law et al., 2006).We therefore coupled LfcinB6 to R7 via a glycine–glycine linker in order to produce a putative membrane-penetrating form of LfcinB6 that we refer to as MPLfcinB6. The effect of MPLfcinB6 on T-leukemia cells, B-lymphoma cells, and normal activated T lymphocytes, as well as the mechanism of cancer cell-selective cytotoxicity was determined.

Materials and methods

Cell culture

Jurkat T-leukemia cells, Raji B-lymphoma cells, and Ramos B-lymphoma cells were purchased from American Type Culture Collection (Manassas, VA). CCRF-CEM T-leukemia cells were kindly provided by Dr W. Gait (University of Alberta, Edmonton, AB). Dominant-negative caspase-9-expressing Jurkat cells and empty vector transfected-Jurkat cells were a kind gift from Dr. R.C. Bleackley (University of Alberta, Edmonton, AB). Caspase-8-deficient Jurkat cells were generously provided by Dr. C. Hao (Emory University, Atlanta, GA). Normal human T cells provided by Dr. Jean Marshall (Dalhousie University, Halifax, NS) were activated with 5 mg/ml concanavalin A (Sigma-Aldrich, Oakville, ON) for 24 h prior to use in experiments. All cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere in RMPI-1640 medium (Sigma-Aldrich) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 5 mM HEPES, all of which were purchased from Invitrogen (Burlington, ON). Cancer cell lines were passaged as required, and cell viability was assessed by trypan blue exclusion to be at least 90% prior to use in experiments.

Reagents

LfcinB (FKCRRWQWRMKKLGAPSITCVRRAF), LfcinB6 (RRWQWR), MPLfcinB6 (RRRRRRRGGRRWQWR), and control MPLfcinB6 (RRRRRRR GGNNGQGN) were purchased from Dalton Chemical Laboratories Inc. (Toronto, ON) or American Peptide Co. (Sunnyvale, CA) at 95% or greater purity. Peptide stocks were prepared in serum-free RPMI-1640 medium and were stored at −80 °C. All experiments with peptides were conducted in medium containing 0.5% FBS to limit peptide degradation by serum proteases. Reduced glutathione (GSH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and fluorescein isothiocyanate (FITC)-dextran were from Sigma-Aldrich. Pan-caspase inhibitor z-VAD-fmk and cathepsin B inhibitor z-FA-fmk were purchased from EMD Biosciences (San Diego, CA). Dihydroethidium (DHE) and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) were from Molecular Probes (Eugene, OR).

Cell viability assays

Cell viability was assessed by measuring MTT reduction by living cells (Mosmann, 1983) or by using the JAM test to measure DNA fragmentation (Matzinger, 1991). T-leukemia cells, B-lymphoma cells (5×10^4 cells/well), or normal activated human T cells (2.5×10^5 cells/well), were seeded, in quadruplicate, into 96-well flat-bottom tissue culture plates and cultured under the indicated conditions for the indicated periods of time. MTT assays and JAM tests were then performed as previously described (Richardson et al., 2009).

Mitochondrial transmembrane potential ($\Delta\psi_m$) measurement

Altered $\Delta\psi_m$ was assessed by flow cytometric analysis of cells stained with $DiOC_6$ (Zamzami et al., 1995), as previously described (Mader et al., 2007). Briefly, 5×10^5 T-leukemia or B-lymphoma cells were cultured under the indicated conditions for the indicated periods of time, then $DiOC_6$ (40 nM) was added to the samples 15 min prior to analysis.

Measurement of ROS production

ROS production was assessed by flow cytometric analysis of cells stained with DHE (Perticarari et al., 1991), as previously described (Mader et al., 2005). Briefly, 5×10^5 T-leukemia or B-lymphoma cells were cultured under the indicated conditions for the indicated periods of time, then DHE (2.5 μM) was added to the samples 15 min prior to analysis by flow cytometry.

Cell membrane damage assessment

Flow cytometric analysis of PI and FITC-dextran uptake, as described previously (Richardson et al., 2009), was used to assess damage to the cell membrane of peptide-treated cells.

Scanning electron microscopy

Peptide-mediated membrane damage was visualized by scanning electron microscopy, as previously described (Hilchie et al., 2011). Briefly, Jurkat T-leukemia cells (5×10^5) were seeded into 24-well flat-bottom tissue culture plates containing poly-L-lysine-coated sterile circular coverslips and were incubated for 2 h to promote cell adhesion. Cells were then cultured under the indicated conditions for 30 min and prepared for analysis.

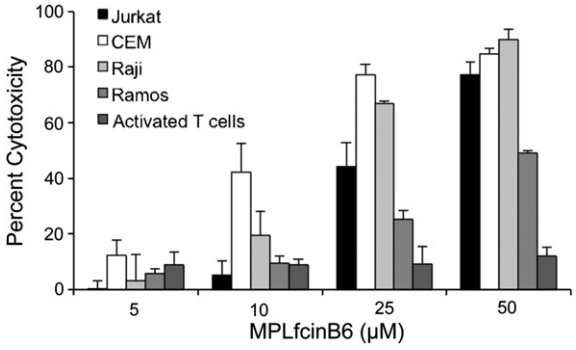

Fig. 1. MPLfcinB6 kills T-leukemia and B-lymphoma cells but spares normal T cells. Jurkat or CEM T-leukemia cells, Raji or Ramos B-lymphoma cells, or activated normal human T cells were cultured in the presence of the indicated concentrations of MPLfcinB6 for 24 h. Percent cytotoxicity, relative to cells cultured in medium alone, was determined by MTT assay. Data shown are the mean of at least 3 independent experiments ± SEM. Differences between MPLfcinB6-treated cancer cells, but not normal T cells, were significant by ANOVA ($p < 0.0001$).