Induction of Cancer Cell Death by Self-assembling Nanostructures Incorporating a Cytotoxic Peptide

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Abstract

Nanotechnology offers novel delivery vehicles for cancer therapeutics. Potential advantages of nanoscale platforms include improved pharmacokinetics, encapsulation of cytotoxic agents, enhanced accumulation of therapeutics in the tumor microenvironment, and improved therapeutic structures and bioactivity. Here, we report the design of a novel amphiphilic molecule that self-assembles into nanostructures for intracellular delivery of cytotoxic peptides. Specifically, a cationic α-helical (KLAKLAK)₂ peptide that is known to induce cancer cell death by membrane disruption was integrated into a peptide amphiphile (PA) that self-assembles into bioactive, cylindrical nanofibers. PAs are composed of a hydrophobic alkyl tail, a β-sheet forming peptide, and a bioactive peptide that is displayed on the surface of the nanofiber after self-assembly. PA nanostructures that included (KLAKLAK)₂ were readily internalized by breast cancer cells, in contrast to the (KLAKLAK)₂ peptide that on its own was not cell permeable. (KLAKLAK)₂ nanostructures, but not the peptides alone, also induced breast cancer cell death by caspase-independent and Bax/Bak–independent mechanisms associated with membrane disruption. Significantly, (KLAKLAK)₂ nanostructures induced cell death more robustly in transformed breast epithelial cells than in untransformed cells, suggesting a degree of tumor selectivity. Our results provide proof-of-principle that self-assembling PAs can be rationally designed to generate nanostructures that can efficiently deliver cytotoxic peptides to cancer cells. Cancer Res; 70(8); OF1–7. ©2010 AACR.

Introduction

Nanotechnology is a multidisciplinary field providing a unique platform to create a new generation of cancer therapeutics with enhanced efficacy and diminished toxicity (1). Nanoscale drug delivery has many advantages over traditional chemotherapy, including improved drug solubility and pharmacokinetics. The enhanced permeability of tumor vasculature results in passive accumulation of nanoparticles in tumors. Therapeutic cargo, imaging agents, and tumor-targeting epitopes can be multiplexed into single nanostructures, thereby engineering controlled tumor delivery of these agents.

Recent studies have shown the utility of peptides designed to activate cell death in cancer cells. One promising strategy uses cationic peptides, such as (KLAKLAK)₂ (abbreviated “KLAK”), as membrane-disrupting agents. In the presence of membranes, this peptide forms an amphipathic α-helix, with the charged and nonpolar residues on opposite faces (2). Because cells do not efficiently internalize KLAK peptide, it is typically fused with cell-penetrating moieties or receptor ligands (3–6). With facilitated uptake, KLAK and other cationic peptides disrupt mitochondrial and/or plasma membranes and initiate caspase-dependent and/or caspase-independent cell death, respectively, reducing tumor burden in vivo (3–7).

Despite potential utility, peptides have several shortcomings, including poor cell penetration, immunogenicity, and rapid in vivo degradation (8). We postulated that incorporating peptides into nanostructures might be an effective strategy to overcome these deficiencies. To this end, we incorporated the KLAK peptide into a novel class of peptide amphiphiles (PA) developed in our laboratory (9, 10). PA molecules have three domains: a hydrophobic alkyl tail, a β-sheet promoting sequence, and a bioactive peptide...
domain. In biological fluids, the self-assembly of PAs into high-aspect-ratio nanostructures is driven by electrostatic screening of charged residues, hydrophobic collapse of alkyl tails into the fiber core, and intermolecular hydrogen bonding, particularly in the β-domain. These nanofibers surface-display peptide segments at high density and have been found to be highly bioactive. PA nanofibers have been used to promote functional recovery after spinal cord injury and angiogenesis (11). Recently, the high aspect ratio of PAs was shown to exhibit greater bioactivity relative to spherical aggregates of similar molecules (12). A major promise of PA nanostructures is the potential for drug-free, biodegradable therapeutics. We report here that a PA incorporating the KLAK peptide self-assembles into nanostructures that are readily internalized by cancer cells and induce cell death, thereby supporting the potential utility of PA-based cancer therapeutics.

Materials and Methods

**Material synthesis and characterization.** Peptides and PAs were prepared using standard fluorenylmethoxycarbonyl solid-phase peptide synthesis methods and were purified by reverse-phase high-performance liquid chromatography. Amino acid analyses were done by Commonwealth Biotechnologies. Circular dichroism (CD) measurements were done on a Jasco CD Spectrometer J-715. PAs were imaged using a JEOl 1230 transmission electron microscope. A more detailed description of the materials is included in Supplementary Data.

**Cell culture and reagents.** SKBR-3 cells (American Type Culture Collection) were grown in α-MEM with 5% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 IU/mL penicillin-streptomycin, 1 mmol/L sodium pyruvate, 20 mmol/L nonessential amino acids, HEPES, and 50 μmol/L β-mercaptoethanol. MDA-MB-231 cells (gift of Jennifer Koblinski, Northwestern University, Chicago, IL) were maintained in DMEM/F-12 with 2x nonessential amino acids, 5% FBS, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamate, and 100 IU/mL penicillin-streptomycin. Wild-type and Bak<sup>−/−</sup> Bax<sup>−/−</sup> mouse embryonic fibroblasts (MEF), a gift of Craig Thompson (University of Pennsylvania, Philadelphia, PA; ref. 13), were maintained in DMEM with 10% FBS, 2 mmol/L L-glutamine, and 100 IU/mL penicillin-streptomycin. MCF-10A cells stably expressing H-RasV12 or empty vector (14) were maintained in DMEM/F-12 with 5% horse serum, 20 ng/mL epidermal growth factor (Sigma), 0.5 mg/mL hydrocortisone (Sigma), 100 ng/mL cholera toxin (Sigma), 10 μg/mL insulin (Sigma), and 100 IU/mL penicillin-streptomycin. All cells were grown at 37°C in 5% CO<sub>2</sub> atmosphere. Unless otherwise specified, reagents were obtained from Invitrogen. Lexitremumab was provided by Robin Humphreys (Human Genome Sciences, Rockville, MD; ref. 15).

**Confocal microscopy and flow cytometry.** Images were taken with a Nikon C1 confocal microscope. Flow cytometry was done using a Becton Dickinson FACSCalibur or a Dako-Cyomation CyAn.

**Immunoblotting.** Cells were lysed in modified radioimmunoprecipitation assay buffer (50 mmol/L Tris, 0.1% SDS, 150 mmol/L NaCl, 0.5% sodium deoxycholate, and 1% NP40) containing 1 mmol/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma). Lysates were run on SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes, and proteins were detected by immunoblotting using antibodies against cleaved caspase-3 (Cell Signaling) or tubulin (Sigma).

**Cell viability.** Cell viability was measured using the MTS-based CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s protocol. Trypan blue (0.04%; Sigma) exclusion was also used to identify viable cells with intact plasma membranes.

** Annexin V assay.** Annexin V-positive cells were identified using the Annexin-PE Apoptosis Detection Kit I (BD Bioscience) according to the manufacturer’s instructions except that 4′,6-diamidino-2-phenylindole (DAPI) was used.

**Membrane potential assays.** Cells were labeled with 50 nmol/L MitoTracker Red and DiOC<sub>5</sub>(3) (Invitrogen) to stain mitochondrial and plasma membranes, respectively, according to the manufacturer’s instructions. Fluorescence was measured at 25°C using a DakoCyomation CyAn.

**Transmission electron microscopy.** Images were obtained using a JEOL 1220 microscope. Cells were fixed in 2% glutaraldehyde/0.1 mol/L sodium cacodylate, treated with 2% osmium tetroxide, and stained with uranyl acetate/lead citrate (Electron Microscopy Sciences).

**Statistical analysis.** Statistical significance was assessed by two-way ANOVA with Bonferroni post-tests using GraphPad Prism 5.0b software.

Results and Discussion

The central component of this nanotechnology platform is the KLAK PA (Fig. 1A; Supplementary Fig. S1). KLAK PA monomers self-assembled in PBS into nanostructures 100 to 900 nm in length and 8 to 10 nm in diameter (Fig. 1B). Because the membrane-disrupting activity of the KLAK peptide (Supplementary Fig. S2) depends on its α-helical conformation (3), we examined the secondary structure of KLAK-containing molecules. The CD spectrum of KLAK PA showed helical structure, whereas KLAK peptide was predominantly random coil (Fig. 1C). We next examined the uptake of fluorescein-labeled KLAK PA (Supplementary Fig. S3) and KLAK peptide (Supplementary Fig. S4) by SKBR-3 breast cancer cells. KLAK PA, but not peptide, was internalized (Fig. 1D). Hence, incorporating the KLAK epitope into a PA stabilizes its helical conformation and facilitates its intracellular delivery.

To determine the cytotoxicity of KLAK PA, we treated human MDA-MB-231 and SKBR-3 breast cancer cells for 24 hours with varying concentrations of KLAK PA or KLAK peptide and measured cell viability by MTS assay (Fig. 2A). The IC<sub>50</sub> of the KLAK PA was 6.04 μmol/L (95% confidence interval, 5.06–7.21 μmol/L) and 5.67 μmol/L (95% confidence interval, 5.30–6.26 μmol/L) in MDA-MB-231 and SKBR-3 cells, respectively, whereas the KLAK peptide was inactive. A

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scrambled KLAK peptide sequence designed to partially disrupt the amphipathic helix was incorporated into a PA (Supplementary Fig. S5), which formed nanofibers with reduced helical structure (Supplementary Fig. S6) and diminished cytotoxicity compared with the KLAK PA (Fig. 2A).

To investigate cell death mechanism(s), breast cancer cells were incubated with PBS, KLAK peptide, KLAK PA, or scrambled KLAK PA for 18 hours. Consistent with the cell viability results, KLAK PA, but not peptide, robustly induced Annexin V-positive cells, whereas scrambled KLAK PA was less effective (Fig. 2B). The majority of cells treated with KLAK PA were both Annexin V and DAPI positive, even when treated for only 1 hour (data not shown), suggesting that these nanostructures rapidly compromise plasma membrane integrity. Within minutes of adding KLAK PA to cells, the plasma and mitochondrial membrane potentials were reduced, suggesting membrane disruption (Fig. 2C, left). To assess the role of oxidative stress in KLAK PA–mediated cell death, cells were pretreated for 1 hour with 3 mmol/L N-acetyl cysteine (NAC). NAC partly attenuated cell death induced by KLAK PA but not that by the proapoptotic TRAIL receptor-2 monoclonal antibody lexatumumab, which served as a positive control (Fig. 2C, right; ref. 15). Consistent with reports showing enhanced sensitivity of cancer cells to cationic peptides due to increased negatively charged membrane phospholipids (16), we observed that MCF-10A breast cells transformed by H-RasV12 were more sensitive to KLAK PA–induced cell death than were the untransformed MCF-10A-vector cells (Fig. 2D). These results suggest that the KLAK PA has some tumor selectivity.

To determine whether KLAK PA–induced cell death was caspase dependent, we incubated MDA-MB-231 cells with...
KLAK PA in the presence of z-VAD-fmk. Although z-VAD-fmk inhibited lexatumumab cytotoxicity, it had no effect on cell death induced by KLAK PA or scrambled KLAK PA (Fig. 3A and B). Consistent with these findings, lexatumumab, but not KLAK PA, induced caspase-3 processing to its active subunit (Fig. 3C). Moreover, wild-type and Bax−/−/Bak−/− double knockout (DKO) MEFs were equally susceptible to KLAK PA cytotoxicity (Fig. 3D). Thus, KLAK PA induces caspase-independent and Bax/Bak− independent cell death.

To investigate ultrastructural changes, MDA-MB-231 cells were treated with PBS, KLAK peptide, or KLAK PA and examined by transmission electron microscopy (TEM). Cells treated with KLAK PA did not exhibit apoptotic morphology, but rather had distorted plasma membranes, abundant fiber-like structures (arrows), and lysosome-like structures (arrowheads) and were largely devoid of mitochondria (Fig. 4A). These changes were not observed in cells treated with KLAK peptide (Fig. 4B) or PBS (Fig. 4C). We also observed that KLAK PA was much more efficient than the scrambled PA at permeabilizing cell membranes as determined by trypan blue staining (Fig. 4D), confirming that KLAK PA nanostructures induce cell death by disrupting cell membranes.

We have shown that PAs can be rationally designed to self-assemble into nanostructures that deliver cytotoxic peptides to cancer cells. Specifically, incorporating the KLAK peptide...
into PA nanostructures stabilizes its bioactive α-helical conformation and renders the peptide cell-permeable. Additionally, KLAK PA nanostructures potently induce caspase-independent and Bax/Bak-independent cell death in breast cancer cells by lysing plasma membranes, a finding consistent with some reports on the cytotoxic mechanism of cationic peptides (6, 7). Moreover, KLAK PAs induce cell death more robustly in transformed breast epithelial cells than in untransformed cells, suggesting some tumor selectivity. Although others have recently reported PA-based platforms for cancer, the cytotoxic mechanisms of these nanostructures have yet to be fully characterized (17, 18). From a therapeutic standpoint, nanostructures will likely have to be PEGylated and/or tumor targeted to maximize their efficacy and reduce systemic toxicity in vivo. Having already shown the feasibility of co-assembling PAs (19), we will focus future

Figure 3. KLAK PA nanostructures induce caspase-independent cell death. A, MDA-MB-231 cells were incubated for 24 h with KLAK PA with or without 50 μmol/L z-VAD-fmk. Lexatumumab (1 μg/mL) served as a positive control. Cell viability was measured by MTS assay. B, MDA-MB-231 or SKBR-3 cells were incubated for 24 h with PBS, 1 μg/mL Lexa, or 10 μmol/L KLAK PA or scrambled KLAK PA with or without 50 μmol/L z-VAD-fmk. The percentage of Annexin V-positive cells was measured. C, MDA-MB-231 cells were treated with 1 μg/mL Lexa or 10 μmol/L KLAK PA for 60 and 120 min. Active caspase-3 was detected by immunoblotting. D, wild-type (WT) and Bax−/−/Bak−/− double knockout (DKO) MEFs were incubated for 24 h with KLAK PA. Cell viability was measured by MTS assay (top). The percentage of Annexin V-positive cells was determined (bottom). In all graphs, data are mean ± SEM (n = 3 or 4). ***, P < 0.001, for the indicated comparisons.
studies on constructing hybrid nanofibers by co-assembly of KLAK PA, PEGylated PA, and tumor-targeting PAs. The high aspect ratio of PA nanofibers may represent a therapeutic advantage over traditional spherical nanoparticles because fibrillar micelles have prolonged circulation time \textit{in vivo}\cite{20}. An additional advantage is that PAs are biodegradable vehicles, disintegrating into amino acids and lipids\cite{11}. Collectively, our findings suggest that PAs represent a promising nanotechnology platform for cancer therapy.

**Disclosure of Potential Conflicts of Interest**

S.I. Stupp: employment, Nanotope, Inc. The other authors disclosed no potential conflicts of interest.
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References

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