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Potential-dependent membrane permeabilization and mitochondrial aggregation caused by anticancer polyarginine-KLA peptides

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ABSTRACT

The anticancer activity of the polycationic peptide $(KLAKLAK)_2$, as a possible mitochondria-damaging agent, named KLA (L-form) or kla (D-form), has been increased by the fusion with hepta-arginine cell delivery vectors r7 and R7 (peptides r7-kla and R7-KLA, respectively), as shown in the literature. We demonstrated that 3.6 μM r7-kla or R7-KLA, but not kla, caused significant permeabilization of the inner and the outer membranes of energized rat liver mitochondria. In addition, r7-kla or R7-KLA induced mitochondrial aggregation, thus causing the inhibition of metabolic activity. Potential-dependent mechanism of permeabilization of the inner mitochondrial membrane by these peptides was also observed for the plasma membrane of red blood cells. The obtained results suggest that polyarginine cell delivery vectors of anticancer polycationic peptides not only increase their direct potential-dependent permeabilization of biological membranes, but also create the capacity to cause aggregation of mitochondria, as a new mechanism of cytotoxic action of these peptides.

Introduction

Natural cationic amphipathic peptides are involved in antimicrobial defense system of humans, animals and plants [1–5]. Many antimicrobial peptides such as magainins, cecropins, melittins and others have also been found to have the ability to kill cancer cells [6–11]. Various artificial polycationic peptides have been designed [12–15] with the aim to retain or enhance the selectivity of natural antimicrobial peptides like magainins [16–17], cecropins [18] and other to kill bacteria, while preserving low toxicity to mammalian cells. Moreover, several artificial peptides enriched in KLA sequences and having antimicrobial activity [12,19] revealed pro-apoptotic action against cultured cancer cells and solid tumors [20–22]. One of them, the 14-amino-acid peptide $(KLAKLAK)_2$, named KLA for its L-form and kla for its D-form [22], demonstrated 100 times higher efficiency in killing bacteria than eukaryotic cells [12].

The kla[1] peptide has been converted into short peptides selectively toxic to angiogenic endothelial cells by its conjugation to the "tumor-homing" cyclic peptide CNGRC or to the double-cyclic peptide ACDCRGDCFC through a glycinylglycine bridge to guide kla to targeted cells and allow its receptor-dependent internalization [20]. Kelly and Jones [23] isolated a 9-amino-acid peptide, CPIEDRPMC (RPMrel) specifically detecting HT29 colon carcinoma cells and conjugated it to kla. The obtained conjugate internalized and selectively killed HT29 cells. Highly selective cell internalization of kla has been achieved via its conjugation to monoclonal antibodies, allowing efficient killing of malignant hematopoietic cells at the low nanomolar range of conjugate concentrations [24]. The peptide KLA, conjugated to the antibody of prostate-specific membrane antigen, was two orders of magnitude more potent than the un-conjugated peptide in killing of LNCaP colon cancer cells [25].

To increase anticancer activity of KLA peptide, its cell delivery has also been enhanced by conjugation to a protein transduction domain PTD-5 (a 12-mer peptide with five arginine and two lysine residues) [21,26], or to a hepta-arginine cell penetrating peptide [22]. The antimicrobial kla or KLA fragments of these conjugates have been suggested to be responsible for specific targeting and disruption of mitochondria in eukaryotic cells, because kla caused a decrease of apparent light absorbance in the suspension of isolated rat liver mitochondria [20]. Nevertheless, the light dispersion decrease might also be caused by aggregation of mitochondria [27,28].

The study of mitochondrial and, in general, membrane aspects of the action of known anticancer peptides is very important in order to clarify the mechanism of their pro-apoptotic activity and to design new peptides with enhanced tumor selectivity and efficiency. A direct damage of mitochondria by the peptide kla, for example, has never been studied enough to explain cytotoxic activity of anticancer peptides based on it.

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[1] *Abbreviations used:* kla, D-peptide klaklakklaklak; r7-kla, D-peptide rrrrrrr-gg-klaklakklaklak; R7-KLA, L-peptide RRRRRRR-GG-KLAKLAKKLAKLAK; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl-cyanide-*p*-trifluoromethoxy phenylhydrazone.

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In this work we studied the permeabilization of the inner and the outer membranes of isolated rat liver mitochondria, and of the plasma membrane of red blood cells (RBC) by kla peptide and by two conjugated peptides, rrrrrrr-gg-klaklakklaklak (named as r7-kla) and RRRRRRR-GG-KLAKLAKKLAKLAK (named as R7-KLA) [20], formed by a fusion of kla and KLA with cell penetrating vectors r7 and R7, respectively, [29,30]. These conjugated peptides have been reported to be active against various cancer cell lines in culture and solid HT-1080 fibrosarcoma *in vivo* [20]. In addition, we compared mitochondrial effects of these polycationic peptides with those caused by the novel antimicrobial peptide BTM-P1 derived from the amino-acid sequence of the protoxin Cry11Bb1 [31–34]. Polycationic peptide BTM-P1 contains the fragments KYL, LAK, ALK and AKLK [31], as well as a double motif AXXXA (AXXXAXXXA) [34] favoring peptide oligomerization within the biomembranes [35].

The data obtained in this work demonstrated that r7-kla and R7-KLA permeabilize the inner membrane of isolated rat liver mitochondria at concentrations that are enough to kill cancer cells [20]. The permeabilizing activity of these peptides was manifested in activation of state 4 respiration, in a decrease of potential-dependent level of mitochondrial NAD(P)H, as well as in swelling of mitochondria and increased permeability of the outer mitochondrial membrane to cytochrome *c*, according to a significant increase in the rate of rotenone-insensitive cytochrome *c*-dependent oxidation of external NADH [36]. An essentially new observation was that the peptides r7-kla and R7-KLA caused the aggregation of mitochondria. Membrane permeabilizing activity of these peptides was strongly dependent on the inner membrane potential of mitochondria. In experiments with red blood cells, artificially generated high plasma membrane potential significantly increased cell permeabilizing activity of r7-kla and R7-KLA. With this respect, the unmodified kla peptide, at the same concentration as r7-kla or R7-KLA, had almost no effect on the membrane permeability of mitochondria or RBC. Short reports related to these data were published earlier [37,38].

In general, the obtained results suggest that the polycationic cell delivery vectors of anticancer peptides increase not only their cell delivery, but also enhance direct potential-dependent permeabilization of mitochondrial and plasma membranes and create the capacity to induce mitochondrial aggregation that might be a new mechanism of cytotoxic action of anticancer polycationic peptides.

Materials and methods

Materials

The chemicals of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA), valinomycin was from Merck. The peptide BTM-P1 was synthesized by Fundación Instituto de Inmunología de Colombia (Bogota, Colombia) by the solid phase method [39], according to the amino-acid sequences designed by us. A *p*-methyl benzhydrylamine-resin (0.7 Meq/g), *t*-Boc amino acids (Bachem, USA) and low–high cleavages were used in the process [40]. The peptide was purified by high-pressure liquid chromatography, analyzed by mass spectrometry (MALDI-TOF), lyophilized and kept as a powder and as a water solution at 4 °C before use. The peptides kla, r7-kla and R7-KLA, the same as studied in [22], were generously provided by the Center for Molecular Imaging Research, Massachusetts General Hospital, Harvard Medical School.

Isolation of mitochondria

Liver mitochondria from male Sprague Dawley rats (4–6 months of age, starved overnight) were isolated by the method of differential centrifugation as described earlier [41]. After twofold washing in medium containing 210 mM mannitol, 70 mM sucrose, 50 μM EGTA–KOH, 0.3 mg/ml BSA, 10 mM Hepes–KOH, pH 7.2, mitochondria were finally resuspended in 1 ml of the same medium without BSA. Protein content was evaluated by a fast volumetric test described earlier [42].

Isolation of red blood cells

RBC of Sprague Dawley rats were isolated by centrifuging of approximately 20% blood in medium of 120 mM NaCl, 10 mM EDTA and 5 mM sodium citrate, pH 7.4, at 1000*g* for 10 min [34]. The RBC residue was resuspended in 150 mM NaCl, 5 mM Tris–HCl, pH 7.4, and the sample was centrifuged at 1000*g* for 10 min. The last procedure was repeated twofold and the final residue was resuspended in 150 mM NaCl, 10 mM glucose and 5 mM Tris–HCl, pH 7.4, to the final hematocrit of 20%.

Measurement of respiration

The rate of oxygen consumption was measured using Clark-type oxygen electrode as described earlier [36]. Incubation medium was composed of 100 mM sucrose, 75 mM KCl, 5 mM potassium phosphate, 20 μM EGTA-KOH, pH 7.2 (SKPE medium) and supplemented with 5 mM potassium succinate where indicated. Mitochondria were added to the final concentration of 0.5 mg protein/ml. To inhibit the respiratory chain and to simultaneously uncouple the mitochondrial respiration, 2.5 μM rotenone, 0.5 μM antimycin A, 0.5 μM myxothiazol as inhibitors and 1 μM FCCP as an uncoupler (RAMF) were added to the mitochondrial suspension. The rate of oxidation of external 0.6 mM NADH was measured after the addition of 10 μM cytochrome *c*, to evaluate the outer membrane permeability to cytochrome *c*, and after subsequent addition of 0.5 mM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), to estimate the maximum activity of external pathway of NADH oxidation in the conditions when it is not limited by the rate of intermembrane electron transport [36].

Monitoring of the inner membrane potential of mitochondria and of the plasma membrane potential of red blood cells

The inner membrane potential of mitochondria was monitored by potential-sensitive fluorescent probe safranin O (520 nm excitation, 580 nm fluorescence) as described in [43] using Aminco-Bowman Series 2 Luminescence Spectrometer. Mitochondria were added at the concentration of 0.5 mg protein/ml into SKPE medium supplemented with 5 mM potassium succinate and 10 μM safranin O. The plasma membrane potential of RBC was monitored by potential-sensitive fluorescent probe $DiOC_6(3)$ [44]. RBC were added into the medium composed of 150 mM NaCl, 0.1 mM KCl, 5 mM Tris–HCl, pH 7.4 (0.2% final hematocrit) approximately 1 min after the addition of 2 μM $DiOC_6(3)$. The potential-dependent capture of this cationic probe by RBC led to a decrease of fluorescence intensity (444 nm excitation, 501 nm fluorescence).

Monitoring of the redox state of mitochondrial pyridine nucleotides and evaluation of the rate of ATP synthesis

The level of the reduced form of endogenous pyridine nucleotides in mitochondria, NAD(P)H, was monitored fluorimetrically (450 nm fluorescence) using Aminco-Bowman Series 2 Luminescence Spectrometer. To minimize the influence of light dispersion on fluorescence measurements and to decrease the effect of internal filter, the exciting and emitting light beams were focused on the 1.5 × 1.5 mm corner of the cuvette as described in [45], and the excitation wavelength of 365 nm was selected instead of 340 nm. Mitochondria were added to SKPE medium at the concentration of 0.5 mg protein/ml. For the other additions, please see the