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Antitumor effects of cationic synthetic peptides derived from Lys49 phospholipase A_2 homologues of snake venoms

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Abstract

The effects of two cationic synthetic peptides, derived from the C-terminal region of Lys49 phospholipase A_2 homologues from snake venoms, upon various murine tumor cell lines (B16 melanoma, EMT6 mammary carcinoma, S-180 sarcoma, P3X myeloma, tEnd endothelial cells) were evaluated. The peptides are 13-mers derived from *Agkistrodon piscivorus piscivorus* Lys49 PLA_2 (p-AppK: KKYKAYFKLKCKK) and *Bothrops asper* Lys49 myotoxin II (pEM-2[D]: KKWRWWLKALAKK), respectively, in the latter case with slight modifications and with all-D amino acids. All tumor cells tested were susceptible to the lytic action of the peptides. The susceptibility of tumor cell lines was not higher than that of C2C12 skeletal muscle myoblasts, utilized as a non-transformed cell line control. However, in a murine model of subcutaneous solid tumor growth of EMT6 mammary carcinoma, the intraperitoneal administration of pEM-2[D] caused a tumor mass reduction of 36% ($p < 0.05$), which was of similar magnitude to that achieved by the administration of paclitaxel, an antitumor drug in clinical use. Thus, the C-terminal peptides of Lys49 phospholipase A_2 homologues present antitumor effects that might be of interest in developing therapeutic strategies against cancer.

Keywords: Cationic peptides; Antitumor; Phospholipase A_2; Snake venom; Myotoxin

1. Introduction

Cationic peptides are widely distributed in living organisms, playing a variety of functions. They are often referred to as antibacterial or antimicrobial peptides, due to their well-characterized role in innate immunity against infectious agents (Hancock and Scott, 2000; Boman, 2003). A common property of cationic peptides is their ability to permeabilize biological membranes, an effect which can be selective towards prokaryotes, or can be exerted upon both prokaryotic and eukaryotic cells, depending on the structural features of each peptide (Shin et al., 2000; Glukhov et al., 2005). While most of the studies on cationic peptides have focused on their antimicrobial activity, other biological effects are emerging, and in the past few years the ability of some peptides to affect tumor cells has been reported (Wang et al., 2000; Papo et al., 2003; Okumura et al., 2004; Furlong et al., 2006).

Our laboratory has previously characterized a group of basic phospholipases A_2 (PLA_2) present in snake venoms, which induce necrosis of skeletal muscle fibers at the site of injection (Gutiérrez and Lomonte, 1997). A naturally-occurring subgroup of these PLA_2 myotoxins has been shown to be devoid of enzymatic activity due to critical amino acid substitutions, including the replacement of Asp49 by a lysine, and therefore such variants have been referred to as Lys49 PLA_2 homologues (reviewed by Lomonte et al., 2003a). Despite their lack of catalytic activity, these proteins are still able to induce muscle necrosis *in vivo* and to rapidly lyse a variety of cell types *in vitro*. The protein region responsible for such toxic effects in Lys49 PLA_2 homologues was identified near their C-terminus (Lomonte et al., 1994), and synthetic peptides representing this region can mimic their toxic activities

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(Núñez et al., 2001). These short (13-mer) peptides derived from snake venom PLA_2s present the same general features of the antimicrobial peptides involved in innate immunity, since they include a combination of positively-charged and hydrophobic/aromatic residues, and indeed it was demonstrated that they are able to reproduce the bactericidal activity of the parent toxins (Páramo et al., 1998; Santamaría et al., 2005b).

Based on the recent reports on the antitumor activity of some cationic peptides, the purpose of the present study was to evaluate if synthetic peptides derived from the bioactive C-terminal region of Lys49 PLA_2 homologues from snake venoms could have an effect upon tumor cells. Two peptides were selected, representing the region 115–129 of *Agkistrodon piscivorus piscivorus* Lys49 PLA_2 (Núñez et al., 2001) and of *Bothrops asper* Lys49 myotoxin II (Lomonte et al., 1994), respectively. In the latter case, a modified version of the original myotoxin II peptide, which was optimized for antibacterial activity (Santamaría et al., 2005a), was utilized. Results indicate that tumor cells are susceptible to the lytic action of these short synthetic peptides, and that their growth *in vivo* can be inhibited by peptide treatment.

2. Materials and methods

2.1. Synthetic peptides

Peptides p-AppK (KKYKAYFKLKCKK) and pEM-2[D] (KKWRWWL KALAKK) were synthesized using F-moc solid-phase strategy on Rink amide resin (Walker, 1994), either automatically by a commercial provider (SynPep Inc.; p-AppK) or manually in our laboratory (pEM-2[D]). In both cases the amino-terminus was free, while the carboxy-terminus was amidated. The final purity of peptides was higher than 95% by analytical RP-HPLC on a Vydac C4 column (250 × 4.6 mm) eluted at 1 ml/min with a 0–70% acetonitrile gradient in 0.1% trifluoroacetic acid, and their observed mass spectrometry values matched their expected formula values. The peptides are 13-mers derived from the sequence 115–129 (in the common PLA_2 numbering of Renetseder et al., 1985) of two Lys49 PLA_2 homologues isolated from the venoms of *Agkistrodon piscivorus piscivorus* (p-AppK; Núñez et al., 2001) or *Bothrops asper* (pEM-2[D]; Santamaría et al., 2005b), respectively. In the latter case, slight variations from the original sequence present in *B. asper* myotoxin II were introduced, previously selected for enhanced bactericidal action (Santamaría et al., 2005b). The variations in pEM-2[D] consist in a triple replacement of Tyr by Trp to increase hydrophobicity, and the substitution of single Cys and Pro residues by Ala, to avoid possible dimerization by intermolecular oxidation, and to facilitate synthesis, respectively. In addition, this peptide was synthesized with all-D-amino acids, to avoid proteolytic degradation during *in vivo* experiments. It was previously shown that this D-enantiomer (pEM-2[D]) has the same membranolytic potency as its L-counterpart (Santamaría et al., 2005b). Peptides were stored dry at −20 °C and dissolved in 0.04 M sodium phosphate, 0.12 M NaCl, pH 7.2 buffer (PBS) immediately before use.

2.2. Cell lines

The following tumor cell lines of murine origin, obtained from the American Type Culture Collection, were utilized to study the cytolytic effect of synthetic peptides: B16 melanoma (CRL-6323), EMT6 mammary carcinoma (CRL-2775), S-180 sarcoma (TIB-66), and P3X myeloma (CRL-1580). In addition, tEnd cells, a polyoma virus-transformed cell line of murine capillary endothelial origin (Bussolino et al., 1991) was studied. The murine C2C12 skeletal muscle myoblast cell line (CRL-1772) was included as a control of non-transformed cells, known from previous studies to be a suitable target for the cytotoxic action of parent Lys49 PLA_2 toxins from which synthetic peptides were derived (Lomonte et al., 1999). Cells were grown under a humidified atmosphere with 7% CO_2 at 37 °C, in Dulbecco's-modified Eagle medium (DMEM, Sigma) supplemented with 15% fetal calf serum, 2 mM glutamine, 1 mM pyruvic acid, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml). Cells were grown routinely in 25 cm^2 bottles, and replicated after dispersion with trypsin (1500 U/ml) containing 5 mM EDTA, for 5–7 min at 37 °C. For cytolytic activity determinations, cells were seeded into 96-well plates, at an initial density of approximately $1–4\times10^4$ cells/well, and allowed to grow for 3–5 days, as described.

2.3. Cytolytic activity

After reaching near-confluence in the 96-well plates, cell growth medium was removed by aspiration, and varying amounts of peptides (7.5, 15, 30, 60 and 120 μg/well; corresponding to 27.5, 55, 110, 200, and 440 μM concentrations, respectively) were immediately added to the cultures, in a total of 150 μl of DMEM containing 1% fetal calf serum (Lomonte et al., 1999), in duplicate wells. Cytolysis was determined by measuring lactate dehydrogenase (LDH) activity in cell supernatant aliquots collected after an incubation of 3 h with the peptides at 37 °C. LDH activity was quantified by a kinetic assay at 340 nm (LDH-UV kit, Wiener Laboratories). Control cultures included cells incubated with assay medium alone, or with medium containing 0.1% Triton X-100, in order to establish 0% and 100% reference values for cytolysis.

2.4. Tumor growth inhibition in vivo

EMT6 mammary carcinoma cells (8×10^5/animal) were injected by subcutaneous route into BALB/c mice (initial body weight of 18–20 g). One group of twelve mice was treated with intraperitoneal injections containing 250 μg of pEM-2[D] every 48 h (corresponding to a dose of 12–13 mg/kg of body weight), while a control group ($n = 12$) received similar injections containing only vehicle (PBS) instead. A third group of mice ($n = 7$) was treated intraperitoneally with 90 μg (5 mg/kg body weight) of the antitumor agent Paclitaxel (Taxol®, Bristol-Myers Squibb; Wani et al., 1971), every 48 h. Mice were observed daily for tumor growth and to record total body weight. Thirteen days after the injection of EMT6 cells all animals were sacrificed by CO_2 inhalation, and the solid tumors were excised and weighed. Statistical comparison of differences among the groups was performed by ANOVA, followed by Tukey–Kramer Multiple Comparisons Test, using the GraphPad InStat v.3.07 software.

3. Results

Both synthetic peptides evaluated, p-AppK and pEM-2[D], showed a rapid cytotoxic effect against the different cell lines, as indicated by the release of LDH to the supernatants within 3 h (Fig. 1). Cell damage was also evidenced microscopically, by the drastic morphological alterations in the different cell types (Fig. 2). Previous work reported that a scrambled version of p-AppK is completely devoid of cytolytic activity (Lomonte et al., 2003b), ensuring that the lytic action of these cationic peptides, despite being exerted at relatively high concentrations, cannot be attributed to a non-specific effect.

In general, peptide p-AppK was slightly more potent than pEM-2[D] against the various tumor cell lines, except for the P3X myeloma cells, which where slightly more susceptible to pEM-2[D]. Nevertheless, the differences observed among the various tumor cell types varied within the same order of magnitude, with 50% cytolytic concentration values (IC_{50}) ranging between 50–350 μM for both peptides (Table 1). The *in vitro* cytolytic potency of the synthetic peptides for tumor cell lines was comparable to that observed for the