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Apoptotic human neutrophil peptide-1 anti-tumor activity revealed by cellular biomechanics

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ABSTRACT

Cancer remains a major cause of morbidity and mortality worldwide. Although progress has been made regarding chemotherapeutic agents, new therapies that combine increased selectivity and efficacy with low resistance are still needed. In the search for new anticancer agents, therapies based on biologically active peptides, in particular, antimicrobial peptides (AMPs), have attracted attention for their decreased resistance development and low cytotoxicity. Many AMPs have proved to be tumoricidal agents against human cancer cells, but their mode of action is still controversial. The existence of common properties shared by the membranes of bacteria and tumor cells points to similar lipid-targeting mechanisms in both cases. On the other hand, anticancer peptides (ACPs) also induce apoptosis and inhibit angiogenesis. Human neutrophil peptide-1 (HNP-1) is an endogenous AMP that has been implicated in different cellular phenomena such as tumor proliferation. The presence of HNP-1 in the serum/plasma of oncologic patients turns this peptide into a potential tumor biomarker. The present work reveals the different effects of HNP-1 on the biophysical and nanomechanical properties of solid and hematological tumor cells. Studies on cellular morphology, cellular stiffness, and membrane ultrastructure and charge using atomic force microscopy (AFM) and zeta potential measurements show a preferential binding of HNP-1 to solid tumor cells from human prostate adenocarcinoma when compared to human leukemia cells. AFM also reveals induction of apoptosis with cellular membrane defects at very low peptide concentrations. Understanding ACPs mode(s) of action will certainly open innovative pathways for drug development in cancer treatment.

1. Introduction

As cancer remains a significant cause of morbidity and mortality worldwide [1], it is important to rationally analyze the efficacy of conventional therapies and assess its impact on patient's survival and quality of life [1,2]. One of the inspirational sources for anticancer drug leads is the group of so-called antimicrobial peptides (AMPs) [3,4]. In addition to antimicrobial activity, these natural molecules act as conserved effectors in innate immunity [4,5]. Interestingly, many AMPs have proven anticancer activity against human cancer cells [6–9]. The modes of action of these molecules have been extensively studied [10–14], and they have attracted attention since they combine decreased resistance development with low cytotoxicity. AMPs act mainly on the cell membranes via a non-receptor-mediated pathway; therefore, it is difficult for cancer cells to develop resistance [15], which makes AMPs with anticancer activity desirable molecules to be developed as new chemotherapeutic drugs. In addition to membranolytic action, it has been proposed that anticancer peptides (ACPs) trigger intracellular mechanisms of toxicity in tumors [7,16]. Like AMPs, many ACPs have the ability to translocate cell membranes [17] and reach intracellular targets. The activity and selective cancer cell targeting ability of ACPs rely greatly on the increased negative membrane net charge as well as on differences of membrane fluidity presented by tumor cells when compared to normal tissues [15,18].

Defensins are a group of cationic AMPs that have been isolated from different species [19], being the α- and β-defensins from human origin the most intensively studied [20]. α-Defensins are the human neutrophil peptides, or HNPs, which are major components of the azurophilic granules of neutrophils [21,22] in which concentrations of ~10 mg/ml can be achieved [23]. These 30 amino acid residue peptides are produced and released in response to microbial invasions, rapidly inactivating a large spectrum of potential pathogens, either Gram-positive or Gram-negative bacteria, and yeasts [24]. Their effect in cell division, attraction, and differentiation of immune cells and wound healing has been also described [25]. Importantly, the human neutrophil peptide-1, HNP-1, as for other AMPs, showed simultaneous antimicrobial and anticancer

Abbreviations: AMPs, antimicrobial peptides; ACPs, anticancer peptides; HNP-1, human neutrophil peptide-1; EthD-1, ethidium homodimer-1; R_{ms}, root-mean-square roughness; HNPs, human neutrophil peptides; PS, phosphatidylserine; GAGs, glycosaminoglycans

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activity [26,27]. In addition, HNP-1 has been suggested as a potential prognostic biomarker in cancer [28–34] since it has been detected in epithelial tumors and is associated with tumor necrosis when expressed intratumorally [35,36]. Up-regulated in cancers such as bladder, gastric, and colorectal [29,33,34], the role of HNP-1 on the tumor microenvironment and directly on the cancer cell is still unclear. Although it is known that the HNP-1 damages cell membranes and enters cells [37], the exact mechanism of cell death has remained elusive for more than 20 years.

In this work, we show the effects of HNP-1 on human prostate adenocarcinoma (PC-3) and human acute lymphoblastic leukemia (MOLT-4) cells using spectroscopic techniques and atomic force microscopy (AFM). AFM techniques resolve the cellular ultrastructure at the nanometer scale [38]. In oncology, AFM has been used to reveal details of the cell membrane structure and morphology [39–41], interaction with different molecules [42], and elasticity [40,41,43]. In the present study, results on cellular morphology, stiffness, and biomembrane biophysical properties such as surface density charge and roughness allowed the identification of the cellular structures that are damaged by HNP-1. These damages interfere with cells' ability to migrate and invade different organs after interacting with HNP-1.

2. Materials and methods

2.1. Biological material and reagents

Human neutrophil peptide-1 (HNP-1; Ala-Cys-Tyr-Cys-Arg-Ile-Pro-Ala-Cys-Ile-Ala-Gly-Glu-Arg-Arg-Tyr-Gly-Thr-Cys-Ile-Tyr-Gln-Gly-Arg-Leu-Trp-Ala-Phe-Cys-Cys: Cys^2-Cys^{30}, Cys^4-Cys^{19}, Cys^9-Cys^{29}) was purchased from Bachem. Adherent cancer cell line PC-3 (human prostate adenocarcinoma) and suspension cell line MOLT-4 (human acute lymphoblastic leukemia) were purchased from American Type Culture Collection (ATCC, CRL-1435, and CRL-1582, respectively). RPMI-1640 media, heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin solution, 200 mM glutamine solution, and trypsin EDTA were obtained from Life Technologies. Glutaraldehyde was from Sigma.

2.2. Cell culture

Adherent cancer cell line PC-3 was cultured as a monolayer in RPMI-1640 media supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin and maintained at 37 °C and 5% CO_2 in a humidified environment. MOLT-4 cell line was cultured in suspension in RPMI-1640 media supplemented with 10% FBS and 2 mM glutamine and also maintained at 37 °C and 5% CO_2 in a humidified environment.

2.3. Cell live/dead assay using flow cytometry techniques

The cytotoxicity of HNP-1 against PC-3 and MOLT-4 cells was evaluated using a LIVE/DEAD® Viability/Cytotoxicity Assay Kit (L-3224) obtained from Life Technologies. This kit is based on the use of two fluorescent probes, calcein AM, and Ethidium homodimer-1 (EthD-1) that are sensitive to intracellular enzymatic activity and plasma membrane integrity, respectively. The use of these probes allows discriminating between live and dead cells after interaction with HNP-1. Live cells display green and dead cells red fluorescence, respectively. Calcein AM is a cell permeable probe, which is converted by intracellular esterase activity, ubiquitous in live mammalian cells, to green fluorescent (530 nm) calcein. Dead cells are stained by EthD-1 dye, which binds to cellular DNA of cells with their membrane compromised/permeabilized. Double staining also occurs in dead cells, indicating that some esterase activity remained prior to cell damage and further cell death [44]. Confluent PC-3 cells were washed with phosphate buffer saline (PBS) after trypsinization, while MOLT-4 cells were directly washed with PBS. Cells were counted with a cell counter (Scepter 2.0 from Milipore), diluted to 7×10^5 cell/ml and incubated for 4 h at 37 °C with increasing concentrations of peptide up to 10 μM, previously dissolved in PBS buffer. This incubation was performed in culture medium supplemented without serum. Cells were then washed and suspended in PBS buffer. Cell labeling with calcein AM and EthD-1 was performed according to the manufacturer's instructions. Briefly, 2 μL/ml of calcein AM and 4 μL/ml of EthD-1 stock solution were added to each sample and incubated 20 min at room temperature protected from light before measurement. Samples were loaded into a 96-well microplate, and each well was acquired using a BD LSR Fortessa cell analyzer equipped with a high throughput screening (HTS) module and a 488 nm laser. Each measurement consisted in collecting and recording the events of 180 μL of each sample. Calcein green fluorescence and EthD-1 red fluorescence emission were recorded using 530/30 and 610/20 band-pass filters, respectively. Live and dead control populations were also measured for proper live and dead populations discrimination and gating. Dead cells were obtained by inducing cell death with 70% isopropanol solution. Forward and side scatter data were also collected to evaluate cell damage and morphology changes induced by HNP-1. The percentage of HNP-1-induced cell death/damage was calculated according to control cell population gating and manufacturer's instructions. The peptide concentration required to kill half of the cancer cells (IC_{50}) was calculated by fitting a sigmoidal dose–response function [45] to cell death percentage data as a function of HNP-1 concentration using GraphPad Prism v6.0 analysis software.

Each sample was collected in triplicate and in two different days using independent cellular suspensions.

2.4. Zeta potential measurements of live PC-3 and MOLT-4 cells in the presence of HNP-1

The surface charge density of the cancer cells and the electrostatic attraction of HNP-1 toward them were evaluated using zeta-potential technique. Charged particles that are suspended in solution attract ions with opposite charge to their surface. These ions cover the particle surface and bound to it very strongly, forming the Stern layer. In addition to this layer, a second one is formed where the ions diffuse more freely. When the particle diffuses through the solution, the strongly bound ions move with it, contrary to the ions located in the diffuse boundary. The zeta-potential is the potential that exists at this boundary and can be determined by the electrophoretic mobility of the particles in solution [46]. For these experiments, confluent PC-3 cells were washed with PBS buffer after trypsinization. Cells were diluted to 1×10^5 cells/ml in PBS buffer. MOLT-4 cells were washed with PBS buffer, counted and diluted to 2.5×10^5 cells/ml in PBS buffer. Cellular suspensions with and without HNP-1 (0–10 μM) were dispensed into disposable zeta cells with gold electrodes and allowed to equilibrate for 30 min at 37 °C. A set of 15 measurements (~40 runs each) were performed with a constant voltage of 40 V. Control values were obtained by measuring the surface charge of each cellular suspension in the absence of HNP-1 (0 μM). The complete experiment was carried out at least two times using independent cellular suspensions.

2.5. Atomic force microscopy imaging

AFM images were acquired using a JPK Nano Wizard II (Berlin, Germany) mounted on a Zeiss Axiovert 200 inverted microscope (Göttingen, Germany). The AFM head is equipped with a 15-μm z-range linearized piezoelectric scanner and an infrared laser. PC-3 cells were culture in monolayer on IbiTreat 35 mm μ-dishes from Ibidi at 5×10^4 cell/ml for 2 days. For control images, the culture medium was replaced with new medium supplemented without serum followed by an incubation of 4 h. Since we were interested in observing in detail the membrane structure, we proceeded to cell fixation with glutaraldehyde before imaging. After culture media removal, cells were washed with PBS buffer and then fixed for 10 min at room temperature in 2% glutaraldehyde solution, washed with PBS and sterile mili-Q water,