Suppression of Human Solid Tumor Growth in Mice by Intratumor and Systemic Inoculation of Histidine-Rich and pH-Dependent Host Defense–like Lytic Peptides

Arik Makovitzki, Avner Fink, and Yechiel Shai

Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel

Abstract

Previously, we reported that intratumor or systemic inoculation of a cationic 15-mer, innate immunity-like lytic peptide composed of d- and l-amino acids ([D]-K6L9) caused growth arrest of 22RV1 prostate carcinoma xenografts in a mouse model. However, despite its therapeutic potential, this peptide has significant systemic toxicity at concentrations slightly higher than the therapeutic one. Here, we used the acidic environment created by solid tumors as a trigger to activate anticancer lytic peptides by making them cationic only at low pH levels. We achieved this selectivity by substituting lysines (pKa, ~ 10.5) for histidines (pKa, ~ 6.1) in the parental peptide [D]-K6L9. Histidine is protonated below pH 7. For that purpose, we replaced either three or all six lysines in the parental peptide with histidines to obtain the peptides [D]-K3H3L9 and [D]-H6L9. Interestingly, in vitro experiments showed pH-dependent activity only with [D]-H6L9 mainly toward cancer cell lines. However, both peptides showed reduced systemic toxicity compared with the parental peptide. Intratumor and systemic inoculation of these peptides resulted in a significant decrease in the 22RV1 prostate cancer tumor volume and systemic secretion of prostate-specific antigen in a xenograft mouse model. Moreover, histologic modifications revealed a significant reduction in new blood vessels selectively in tumor tissues after treatment with the peptides compared with the untreated tumors. The lytic mode of action of these new peptides, which makes it difficult for the cancer cells to develop resistance, and their selective and pH-dependent activity make them potential candidates for treatment of solid cancer tumors.

Introduction

The development of an effective therapy for malignant diseases has been hindered by the lack of consistent differences between tumors and normal tissue. Thus, unlike antibiotic treatment of bacteria, it has been difficult to develop therapeutic strategies that have major anticancerous effects without having significant cytotoxicity. One major difference between many solid tumors and the surrounding normal tissue is the nutritional and metabolic environment (1, 2). The functional vasculature of tumors is often inadequate to supply the oxygen demands and nutritional needs of the rapidly proliferating population of cancerous cells. This in turn contributes to the development of anaerobic conditions, under which hydrolysis of ATP results in the production of lactic acid. Moreover, a poor and chaotic tumor vascularization leads to the inefficient washout of the acidic products and further contributes to the development of a chronically acidic extracellular environment (2, 3). The consequence of that phenomenon is that the pH of the extracellular space surrounding solid tumors is significantly lower than the pH of the surrounding normal tissues (1, 3–6). Therefore, in recent years, several attempts have been made to use the solid tumor acidity to develop new pH-dependent approaches to cancer therapy (7–10) or drug delivery (11–14) because this venue seems most promising.

A recent approach under study for cancer treatment is the use of cationic antimicrobial peptides (15–19). These peptides, which have significantly higher toxicity to bacteria than to normal mammalian cells, were shown to exhibit a broad spectrum of cytotoxic activity against cancer cells as well (17, 20, 21). In line with this, we reported that intratumor and systemic administration of a short 15-mer d,L-amino acid peptide ([D]-K6L9-LKLLKKLLKKLLKL-NH2, the underlined letters are L-amino acid) specifically inhibited the growth of primary human prostate carcinomas. The electrostatic attractions between the negatively charged components of the membrane of cancer cells and the positively charged peptide are believed to play a major role in the strong binding of the peptide and its ability to selectively disrupt the membrane of cancer cells (22, 23). However, similar to other membranolytic peptides, higher concentrations of this peptide could damage normal cells and therefore narrow the therapeutic index.

Here, we show the use of the acidic microenvironment created by solid tumors as a trigger to activate anticancer lytic peptides that otherwise are not active under physiologic conditions. For that purpose, we used [D]-K6L9 (22, 23) as a template and substituted either three lysines (pKa, ~ 10.5) or all of them for histidines (pKa, ~ 6.1), a change that should make the peptides positively charged and active primarily at acidic pH values.

Materials and Methods

Cell culture. The CL1 human prostate carcinoma cell line used is an androgen-independent subclone of LNCaP, which was generated by cultivating adenocarcinoma LNCaP cells in charcoal-stripped serum, as previously described (24). The 22RV1 human prostate carcinoma cells are an androgen-independent subclone of the adenocarcinoma prostatic adenocarcinoma CWR22 xenograft (25). The CL1 and 22RV1 [American Type Culture Collection (ATCC)] were grown in RPMI 1640 supplemented with 10% FCS (Biological Industries) and antibiotics. NIH 3T3 mouse fibroblast cell lines (ATCC) were grown in DMEM supplemented with 10% FCS.
fetal bovine serum (FBS), t-glutamine, and antibiotics (Biological Industries). Similarly, OL human foreskin fibroblasts (ATCC; a generous gift from Prof. Menachem Rubinstein, Department of Molecular Genetics, The Weizmann Institute of Science, Rehovot, Israel) and murine Lewis lung carcinoma (LLC) cell lines were maintained in DMEM supplemented with 10% FBS, t-glutamine, and antibiotics.

**Peptide synthesis and purification.** Peptides were synthesized by a 9-fluorenylmethoxycarbonyl solid-phase method on Rink amide MBHA resin (Calbiochem-Novabiochem) by using an ABI 433A automatic peptide synthesizer (Applied Biosystems) followed by peptide cleavage from the resin and purification by reversed-phase high-performance liquid chromatography (>98%). The composition of the peptides was confirmed by mass spectrometry and amino acid analysis.

**Acute toxicity.** Aliquots of medium containing 1 × 10^5 cells (malignant: CL1, 22RV1, and LLC; nonmalignant: NIH 3T3 and OL) were distributed into a 96-well plate (BD Falcon). The following day, the media were replaced with 90 μL fresh culture medium adjusted to pH 7.4 or 5.5 and 10 μL of a solution buffer (adjusted to pH 7.4 or 5.5, respectively) containing different concentrations of the peptides. The plate was then incubated for 24 h before adding to each well 50 μL of 2.3-bis-[2-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) reaction solution (Biological Industries). Viability was determined as described previously (22, 23). The LC50 (the concentration at which 50% of the cells die) for each peptide was obtained from the dose-dependent cell viability curves.

**Membrane permeability studies.** Vesicles composed of phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and cholesterol (4.35/4.35/1.0/3.1, w/w) and entrapped with calcine [60 mmol/L calcine, 10 mmol/L HEPES, and 150 mmol/L NaCl (pH 7.4)] were generated by using the extrusion method as described before (22). Peptides were then added to vesicle suspension (2 mL, 2.4 μmol/L liposomes) adjusted to pH 6 or 7.4. Peptide-induced calcine leakage resulted in an increase in fluorescence (λex = 485 nm; λem = 515 nm; ref. 26), which was monitored.

**Sytox Green uptake assay.** Aliquots of medium containing 1 × 10^5 CL1 cells were distributed into a 96-well plate. The following day, the media were replaced with 90 μL fresh culture medium adjusted to pH 7.4 or 5.5 and 10 μL of a solution buffer (adjusted to pH 7.4 or 5.5, respectively) containing different concentrations of the peptides. Then, 100 μL Sytox Green (Molecular Probes) was added to a final concentration of 1 μmol/L. After 15 min, the cells were examined with a fluorescent microscope (Eclipse, Nikon) applied to a digital camera (C4742-95; Hamamatsu). Excitation was set at 485 nm and emission at 520 nm.

**Inhibition of tumor growth in human prostate carcinoma xenografts.** The s.c. implantation of human prostate carcinoma in mice was done as described previously (27). Briefly, 0.1 mL of androgen-independent 22RV1 human prostate carcinoma cells (5 × 10^6) in Matrigel (BD Biosciences) was inoculated s.c. into the dorsal side of 5- to 6-wk-old nude male mice with an average weight of 20 to 25 g (Harlen). One week after cell implantation, when the tumor diameter reached ~5 mm (we defined this day as day 0), all the peptides or vehicles (Hartmann’s lactate solution at 9mg/kg; 500 μL). This treatment was carried out three times a week (every second day) for a total of nine doses (n = 10 mice per group). Tumor size was measured by a caliper and recorded twice a week. The CanAg prostate-specific antigen (PSA) EIA kit (CanAg Diagnostic) was used to determine total PSA in the mice plasma (27). Tumor size and PSA levels, represented as mean ± SE, were analyzed by Student’s t test. P < 0.05 was considered as statistically significant.

**Histologic and immunofluorescent staining.** Excised tumors were fixed in 4% buffered formaldehyde. Paraaffin-embedded 5-μm sections were stained with H&E and examined using a microscope (Eclipse E800M, Nikon) with a digital camera (DXM1200, Nikon). For immunofluorescent staining, the tumors were fixed with 4% buffered formaldehyde for CD34 epitope labeling (new blood vessels) or zinc fixative perfusion for CD31 epitope labeling (old blood vessels). Paraaffin-embedded 5-μm tumor sections were overlaid with rat anti-mouse CD31 antibody (Pharmingen) or rat anti-mouse CD34 antibody (Cedarlane) against blood vessels. Sections were incubated with bridging biotinylated rabbit anti-rat antibodies (Vector Laboratories) and visualized with streptavidin-conjugated FITC (Jackson ImmunoResearch Laboratories). For quantitative analysis, capillaries, identified by positive staining for CD34 and CD31, were counted and their density was expressed as the percentage of capillaries of the total section area using Image-Pro Plus 4.1 software. To quantify the vessels, 10 nonneocrotic areas at 100 μm^2 per field at ×200 were captured using an Olympus BX-40 microscope (Olympus).

**Results**

**In vitro activity of the peptides toward malignant and normal cells at acidic and physiologic pHs.** Using the template of [D]-K6L9 (22, 23), we synthesized two histidine-containing peptides: [D]-K3H3L9 and [D]-H6L9 (Table 1). The LC50 values of the peptides shown in Table 2 reveal that only [D]-H6L9 has pH-dependent activity. However, all three peptides showed selective activity toward cancer cells (Table 2). Note that the histidine-containing peptides are less active than the parental peptide [D]-K6L9. Nevertheless, the amount administrated to mice is similar to that reported previously for [D]-K6L9.

**Membrane disruption induced by the peptides.** Peptides, at increasing concentrations, were added to a suspension of vesicles (lipid composition of cancer cells; refs. 28, 29) encapsulating calcine (2.5 μmol/L phospholipids) at pH 7.4 or 6. Membrane permeability was measured by monitoring the fluorescence recovery. The level of maximum leakage reached, as a function of the peptide-to-lipid molar ratio, is shown in Fig. 1A. [D]-K6L9 and [D]-H6L9 permeate vesicles at both pHs, whereas [D]-H6L9 was highly active only at pH 6.

**pH-dependent permeation of the cancer cell membrane.** Sytox Green can penetrate into cells when their membrane is disrupted and its fluorescence increases drastically when bound to intracellular nucleic acids. Our data reveal that at a concentration of 3.1 μmol/L (LC50 value), [D]-H6L9 induced an influx of Sytox

<table>
<thead>
<tr>
<th>Peptide designation</th>
<th>Sequence*</th>
<th>Systemic toxicity†</th>
</tr>
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<tbody>
<tr>
<td>[D]-K6L9</td>
<td>LHLLKLLKLLKLLKLLKLLK</td>
<td>Toxic over 8 mg/kg</td>
</tr>
<tr>
<td>[D]-K3H3L9</td>
<td>LKLIKLLKLLKLLKLLKLLK</td>
<td>Toxic over 30 mg/kg</td>
</tr>
<tr>
<td>[D]-H6L9</td>
<td>LHLLKLLKLLKLLKLLKLLK</td>
<td>Toxic over 20 mg/kg</td>
</tr>
</tbody>
</table>

*Bold and underlined letters are D-enantiomers.
†Systemic toxicity was measured by i.v. injection of the peptide dissolved in Hartmann’s lactate solution.
Green into CL1 cancer cells only at acidic pHs. No significant influx was observed at physiologic pHs (Fig. 1B).

**Animal studies.** All animal experiments were performed according to regulations approved by the Institutional Animal Care and Use Committee.

**In vivo acute systemic toxicity.** Acute systemic toxicity was observed after a single i.v. injection of [D]-K3H3L9 at concentrations above 8 mg/kg (n = 5 mice). In contrast, both [D]-K3H3L9 and [D]-H6L9 did not indicate any acute systemic toxicity at doses of up to 30 and 20 mg/kg, respectively (n = 5 mice for each group; Table 1).

**Inhibition of solid prostate cancer growth by intratumor administration of the peptides.** 22RV1 cells were implanted s.c. in mice. One week after tumor implantation, [D]-H6L9 and [D]-K3H3L9 were injected intratumorally at a dose of 1 mg/kg (Fig. 2). A significant inhibition in tumor growth volume was observed during the course of the experiment in mice that were treated with the peptides (Supplementary Fig. S1). In addition, a significant reduction (81%) in dissected tumor weight was measured on the last day of the experiment (Figs. 3 and 4A and B). The reduction in tumor size was accompanied by a marked reduction in 22RV1-secreted PSA levels (Fig. 3 and S4). Histologic examination of dissected tumor sections stained with H&E and CD34 (Figs. 3 and 4C and D, respectively) revealed similar results as those obtained by intratumor administration (see above). Note that CD34 staining used for detection of established microvessel tube densities (CD34 staining) within and around the cancerous tissue revealed that the untreated tumor contained a branched net of newly formed capillary tubes, whereas the treated tumors barely contained newly formed capillary tubes in the adjacent connective tissue (Fig. 2D). However, the newly formed capillary tubes in the adjacent connective tissue were not damaged from the inside the tumors (Fig. 2).

**Inhibition of solid prostate cancer growth by systemic administration of the peptides.** 22RV1 cells were implanted s.c. in mice. One week after implantation, [D]-K3H3L9 and [D]-H6L9 were injected systemically at a dose of 9 mg/kg (Figs. 3 and 4). A significant inhibition of tumor growth was observed during the course of the experiment in mice treated with the peptides (Supplementary Figs. S2 and S3A). In addition, a significant reduction (75% and 72% for [D]-K3H3L9 and [D]-H6L9, respectively) in dissected tumor weight was measured on the last day of the experiment (Figs. 3 and 4F and B). The reduction in tumor size was accompanied by a marked reduction in 22RV1-secreted PSA levels compared with the control group (Supplementary Figs. S2B and S3B). Histologic examination of dissected tumor sections stained with H&E and CD34 (Figs. 3 and 4C and D, respectively) revealed similar results as those obtained by intratumor administration (see above).

### Table 2. Lethal concentration (LC50 in μmol/L) of the peptides at physiologic and acidic pHs detected by XTT assay

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Malignant cells</th>
<th>Nonmalignant cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL1 prostate carcinoma</td>
<td>LLC lung carcinoma</td>
</tr>
<tr>
<td></td>
<td>pH 7.4  pH 6</td>
<td>pH 7.4  pH 6</td>
</tr>
<tr>
<td>[D]-K6L9</td>
<td>2.3  2.3</td>
<td>6.2  6.2</td>
</tr>
<tr>
<td>[D]-K3H3L9</td>
<td>9.3  9.3</td>
<td>12.5  12.5</td>
</tr>
<tr>
<td>[D]-H6L9</td>
<td>12.5  3.1</td>
<td>75   9.3</td>
</tr>
</tbody>
</table>

*NOTE: Results are the mean of three independent experiments, each performed in duplicate.*
capillary tubes revealed no significant difference in the densities of these capillary tubes in treated and untreated tumors (data not shown).

**Discussion**

The important findings in this study are the following: (a) the histidine-containing peptides have significantly reduced systemic toxicity and (b) the entirely substituted histidine peptide has pH-dependent activity. The peptide damaged membranes and cells only at acidic pH (Table 2; Fig. 1). We have shown previously that the selective activity of the parental peptide [D]-K₆L₆ toward cancer cells results predominantly from differences between the membranes of malignant and normal cells (23). Whereas normal cells have mainly zwitterionic membranes on their outer leaflet (30), cancer cells express approximately 3% to 9% more of the acidic phospholipids, phosphatidylserine, in their outer leaflet (31–33). These cells also contain a higher density of negatively charged O-glycosylated mucins compared with normal cells (34, 35). Thus, electrostatic interactions between cationic peptides and the anionic cell membrane components are believed to be a major factor in the selective killing of cancer cells by these peptides. Because the histidines in [D]-H₆L₉ are protonated at acidic pHs, the peptide is not active at physiologic neutral pHs. Indeed, [D]-H₆L₉ is the only peptide that could not permeate model membranes and cancer cells at physiologic pH (Fig. 1). Interestingly, [D]-K₃H₃L₉ has no pH-dependent activity, although it contains a significant number of histidines. This differs from several natural antimicrobial peptides that have pH-dependent activity, although they contain only a few histidines in their sequence (36–47).

The membranolytic activity of the peptides toward model membranes (Fig. 1A) and cell membranes (Fig. 2) supports our assumption that the histidine-containing peptides kill cancer cells by disrupting the cell membrane similarly to [D]-K₆L₆ and other antimicrobial peptides (20, 22, 23, 29). Importantly, substituting lysines for histidines significantly reduced the systemic acute toxicity of [D]-H₆L₉ and [D]-K₃H₃L₉ in treated mice (23), consistent with the reduced membrane permeabilization of their cell membranes (Fig. 2).
toxicity of the peptides (Table 1). Furthermore, topical and systemic administration of these two peptides significantly inhibited 22RV1 prostate carcinoma tumor xenograft growth and lowered the level of PSA known to be secreted by 22RV1 xenografts (25). Note that [D]-K3H3L9 was less cytotoxic than [D]-H6L9. This might be due to the relatively elevated toxicity of the latter in acidic organs besides the tumor.

Previous studies have reported on the use of various techniques to activate anticancer drugs in the presence of the low pH. However, the majority of these techniques focused on drug delivery systems that are pH dependent. Examples include the controlled, pH-dependent release of chemotherapy agents from polymers (12–14) and liposomes (48) as well as by using cell-penetrating peptides (11, 49). Here, [D]-H6L9 possesses three functions: targeting, activation at low pHs, and cytotoxic activity. We expect that owing to the strong membranolytic effect of the peptides, it would be difficult for the tumor cell to select resistant variants. This is similar to what has been found in many cases with bacteria treated with cationic innate immunity lytic peptides (15, 50).

In addition to the potent inhibition of tumor growth by [D]-H6L6 and [D]-K6H6L3, the histology of the tumors treated both intratumorally and via systemic inoculation revealed a significant decrease in vascularization of new capillary tubes compared with untreated mice (Figs. 2D, 3D, and 4D). This could be the result of either the reduced cancer cell density or the direct activity of the peptides toward the sensitive new capillary tubes. The latter is supported by the recent finding that endothelial cells forming the branched vasculature supporting solid tumors express enhanced amount of phosphatidylserine molecules on the outer leaflet of their plasma membrane (51, 52). Treatment of the tumors with the peptides was also accompanied by a reduction in the density of cancer cells and an increase in the amount of newly formed connective tissue within the tumor. This phenomenon correlates with an increased number of cells containing necrotic debris, comprehensive hematomas, and pertinacious liquid found within the cancerous tissue after treatment. Taken together, these results suggest that the observed reduction in the tumor size is only an underestimation of the combined destructive effect of the peptides on the tumor tissue and cells.

In summary, the histidine-rich peptides offer a new class of membranolytic agents for efficient anticancer treatment with...
reduced systemic toxicity. Moreover, the pH-selective activity of [D]-HLM$_4$ may provide a new strategy for treatment of solid tumors within naturally occurring low-pH extracellular environments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

19. Tumor Elimination by a Host Defense–like Lytic Peptide

Acknowledgments

Received 8/5/08; revised 1/14/09; accepted 1/30/09; published OnlineFirst 4/7/09.

Grant support: Israel Cancer Research Funds and Prostate Cancer Research Foundation (United Kingdom). Y. Shai is the incumbent of the Harold S. and Harriet B. Brady Professorial Chair in Cancer Research.

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doi:10.1158/0008-5472.CAN-08-3021

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