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A lactoferrin-derived peptide with cationic residues concentrated in a region of its helical structure induces necrotic cell death in a leukemic cell line (HL-60)

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Abstract: Model studies have shown that peptides derived from the *N*-terminal region of bovine lactoferrin (Lf-B) exhibit antitumor activity against certain cell lines. This activity is due primarily to the peptides' apoptogenic effect. Several reports indicate that cationic residues clustered in two regions of the peptide sequence can be shuffled into one region and thereby increase cytotoxic activity, although the mechanism of this enhanced cytotoxic effect has not been clarified. In this paper, we considered several parameters that determine the mode of cell death after exposure to a native Lf-B derived peptide (Pep1, residues 17–34), and a modified peptide (mPep1) wherein the cationic residues of Pep1 are clustered in a single region of its helical structure. We found that the cytotoxic activity of mPep1 was about 9.6 fold-higher than that of Pep1 against HL-60 cells, as determined by the 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2 H-tetrazolium (MTS) assay. In investigating the expression of phosphatidylserine, we observed that the native peptide (Pep1) caused both apoptotic cell death and necrotic cell death, depending on the concentration of the peptide. In contrast, the action of mPep1 was exclusively characteristic of necrotic cell death. This observation was further confirmed by agarose gel electrophoresis, in which clear ladder-like DNA bands were observed from cells exposed to Pep1, whereas DNA from cells treated with mPep1 produced a smeared pattern. We extended the study by investigating the release of mitochondrial cytochrome *c* into the cytosol, and the activation of caspase-3; both peptides caused the release of cytochrome *c* into the cytosol, and the activation of caspase-3.

These results suggest that Pep1 may kill cancer cells by activating an apoptosis-inducing pathway, whereas mPep1 causes necrotic cell death by destroying cellular membrane structure notwithstanding sharing some cellular events with apoptotic cell death.

Keywords: lactoferrin; peptide; *N*-terminal; apoptosis

INTRODUCTION

Bovine lactoferrin (Lf-B) is an iron-binding glycoprotein consisting of 689 amino acids. The protein has various biological functions such as antimicrobial activity, antivirus activity, anti-inflammatory activity and immune regulation. In particular, lactoferricin (Lfcin-B), a cationic-rich peptide derived from the *N*-terminal region (residues 17–41) of Lf-B, displays higher antimicrobial activity than the native protein [1,2]. Recent evidence additionally indicates that Lfcin-B possesses potent *in vivo* activity against cancer cells [3,4]. Yoo *et al.* have shown that Lfcin-B, but not Lf-B, is a potent inducer of apoptosis in THP-1 human monocytic leukemia cells [5]. In a previous study we have isolated four novel cytotoxic peptides derived from Lf-B: Pep1 corresponding to residues 17–38, Pep2 corresponding to residues 1–16 and 45–48 linked by disulfide bridge, Pep3 corresponding to residues 1–15 and 45–48 linked by disulfide bridge and Pep4 corresponding to residues 1–13 [6]. In contrast, Yang *et al.* designed 14 peptides based on the sequence of the *N*-terminal helical region (residues 14–31) of Lf-B with a view to investigating the relationship between the structure of the designed peptides and their antiproliferation activity against a murine fibrosarcoma cell line (Meth A), a human colorectal adenocarinoma cell line (HT-29), and a human mammary carcinoma cell line (MT-1) [7]. Peptides in which the distribution of amino acid residues was changed by shuffling cationic amino acid residues from the minor cationic sector to the major cationic sector showed enhanced cytotoxic activity. However, little is known about the mechanism by which these shuffled peptides exert their enhanced cytotoxic activity, and even the mode of cell death has not been investigated.

Apoptosis and necrosis are two modes of cell death originally defined by distinct morphological criteria [8]. Classically, apoptosis is induced by a genetic program initiated by expression of immediate early genes and of death effecter proteins that, by a variety of mechanisms, activate cascades of proteolytic enzymes called caspases. In contrast, necrosis is caused by a passive phenomenon induced by ion fluxes and cell explosion or cellular injury. Of these two modes of cell

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death, apoptosis has been considered as a key target for cancer chemo-prevention [9]. The aim of the present article is to clarify the mode of cell death by investigating cell viability, DNA fragmentation, phosphatidylserine expression, release of cytochrome *c* from mitochondria, and caspase-3 activation in promyelotic leukemia cells (HL-60) exposed to Pep1 or mPep1. In this study, mPep1 was designed according to Yang *et al.* [7] by shuffling cationic residues from the minor to the major cationic sector in the helical region of Pep1.

MATERIALS AND METHODS

Cell Culture and Reagents

Human leukemia HL-60 cells were obtained from the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 0.1 mg/ml streptomycin and 100 U/ml penicillin, at 37 °C in a humidified atmosphere containing 5% CO_2. Pep1, corresponding to residues 17–38 of Lf-B, and mPep1, which was designed according to the Lf-B–derived peptide, L5, described by Yang and coworkers [7], were synthesized and HPLC-purified by Sigma-Genosys (Ishikari, Japan). The purity of the peptides was over 95%. All other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) unless otherwise stated.

Cell Treatment and Viability Assay

Pep1 and mPep1 were each dissolved in RPMI-1640 at a concentration of 2.78 mM and diluted with the same culture medium containing 10% FBS. HL-60 cells were incubated with various concentrations of each peptide for different times, as indicated in each assay section. Cytotoxic activities of Pep1 and mPep1 were determined using the Cell Titer 96 Aqueous Cell Viability Assay Kit (Promega, Madison, WI). Briefly, 10 µl of successive twofold diluted solutions of Pep1 or mPep1 were added to individual wells containing 100 µl of HL-60 cell suspension (5×10^5 cells). Then, the plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h incubation, 22 µl of 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2 H-tetrazolium (MTS) working solution was added to each well and the plate was incubated for another 4 h. The amount of resulting formazan was determined by measuring the absorbance at 490 nm using a micro-plate reader.

Necrosis and Apoptosis Assay

Necrotic and apoptotic cells were detected by both propidium iodide (PI) and annexin V labeling using an annexin V-FITC Kit (Immunotech, Marseille, France). Briefly, HL-60 cells (5×10^5 cells) were treated with Pep1 (15–253 µM) or mPep1 (4–126.5 µM) for 24 h. At the end of incubation, the cells were washed with phosphate buffered saline (PBS) buffer and suspended in 100 µl of binding buffer. Annexin V (5 µl) and PI (5 µl) were added to the cell suspension, and the mixture was incubated for 10 min on ice in the dark. The stained cells were analyzed using a FACS can flow cytometer (Model FACS; Calbur, Bectoson, CA). The annexin V-FITC signal was detected using an FL1 (525 nm) detector and the PI signal was monitored using a detector reserved for phycoerythrin emission (FL2, 575 nm).

Analysis of DNA Fragmentation

HL-60 cells (5×10^5 cells) were incubated in the presence of 126 µM Pep1 or 32 µM mPep1 at 37 °C for 6, 12, or 24 h. As a positive control for DNA fragmentation, actinomycin D was added at a concentration of 1 µg/ml. After incubation, cells were collected by centrifugation and washed twice with PBS. The DNA was extracted with an ApoplLadder Ex Kit (Takara Biomedicals, Osaka, Japan). The DNA preparation was subjected to electrophoresis in 1.5% agarose gels, and then the gels were stained with ethidium bromide and photographed with an UV transilluminator.

Western Blot Analysis for Cytochrome *c*

Briefly, 5 ml of cell suspension (5×10^5 cells/ml) was incubated in the presence of 126 µM Pep1 or 32 µM mPep1 at 37 °C for 3, 6, or 12 h. At the end of incubation, the cells were collected by centrifugation and suspended in 1 ml of ice-cold cytosol extraction buffer (provided by a cytochrome *c* Releasing Apoptosis Assay Kit, BioVision Research Products, (Mountain View, CA)). The suspensions were homogenized by repeating ten strokes with a Teflon homogenizer at 0 °C, and then centrifuged at 10 000 *g* for 30 min at 4 °C. The supernatant (400 µl) was concentrated to 50 µl using an Ultrafree-MC 5000 NMWL Filter Unit (Millipore, Bedford, MA); then 40 µl of the concentrate was separated on a 12% gel by (SDS-PAGE) and electroblotted onto a polyvinylamide difluoride (PVDF) membrane using a semidry blotting apparatus (Model AE-7500; Atto Corporation, Osaka, Japan). After blocking the membrane with TBST (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% Tween 20) containing 3% skim milk, the membrane was incubated with anticytochrome *c* antibody included in the cytochrome *c* Releasing Apoptosis Assay kit. The membrane was washed with TBST, incubated with alkaline phosphatase-conjugated antimouse IgG, and the immunoreactive bands were then detected using an Alkaline Phosphatase Substrate Kit (Vector Laboratories, Inc., Burtingame, CA).

Assay for Caspase-3 Activation

Activation of caspase-3 was investigated using a Caspase-3 Intracellular Activity Assay Kit 1 (PhiPhiLux, G1D2, Calbiochem-Novabiochem, San Diego, CA). Briefly, HL-60 cells (5×10^5 cells) were incubated with Pep1 (126 µM) or mPep1 (32 µM) for 12 h. After incubation, the cells were collected by centrifugation and washed twice with PBS. To the cell pellets, 50 µl of 10 µM substrate, 5 µl of FBS, and 1 µl of 1 M Hepes buffer (pH 7.4) were added in the dark and incubated at 37 °C for 1 h in a humidified atmosphere containing 5% CO_2. Cells were washed with ice-cold flow cytometry dilution buffer and centrifuged. Cell pellets were resuspended in 1 ml of flow cytometry dilution buffer and analyzed by flow cytometry at an excitation wavelength of 488 nm using the FL1 channel of a BD instrument.

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RESULTS AND DISCUSSION

Amino Acid Sequence and Helical Wheel Diagram

Figure 1 shows helical wheels of the Pep1 and mPep1 amino acid sequences. A helical wheel drawing tool (CABM Structural Bioinformatics Laboratory, Piscataway, NJ) was used to generate the helical wheels [10]. Previously, Pep1 was identified as a major cytotoxic component in pepsin hydrolytes of Lf-B [6]. In an attempt to increase the cytotoxic activity of Pep1, residues 3, 7, and 14 were replaced with alanine since an alanine-scan experiment on Lf-B showed these residues to be counterproductive for antibacterial activity [11]. The cationic residues in Pep1 are clustered in two separate regions that we termed the major cationic sector (K2, K12, R5, R9) and the minor cationic sector (K11 and R4) (Figure 1(A)). The two cationic residues in the minor sector were scuffled to the major sector by exchanging their positions with lipophilic residues adjacent to the major cationic sector (Figure 1(B)). As a result, the cationic residues in mPep1 were clustered to one side.

Cell Viability Assay

Pep1 and mPep1 produced a marked decrease in the survival of cells following 24 h incubation, with the decrease in cell viability being proportional to the concentration of the peptide (Figure 2). The IC_{50} values for Pep1 and mPep1 calculated from the dose response curves (Figure 2) were 77 and 8 μM, respectively. These

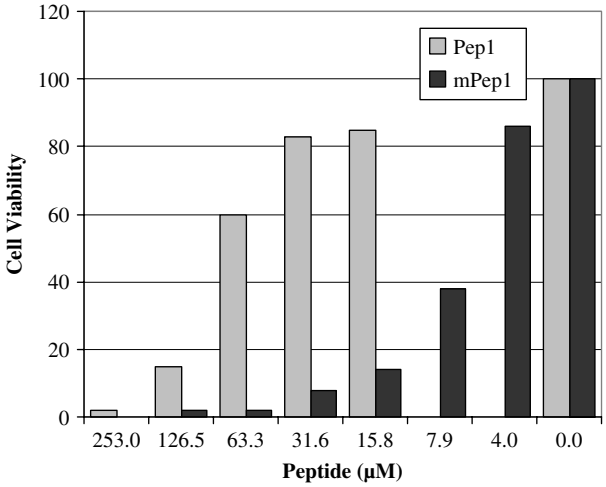

Figure 2 Cytotoxic activity of Pep1 and mPep1 against HL-60 Cells. (A) cytotoxic activity of Pep1. (B) Cytotoxic activity of mPep1. Cytotoxic activity was measured by the MTS assay as described in 'Materials and Methods'.

results show that the cytotoxicity of mPep1 is about 9.6 fold higher than that of Pep1.

Apoptosis and Necrosis Assay

Annexin V is a protein that shows high affinity for phosphatidylserine. Phosphatidylserine is present on the surface of the plasma membrane of apoptotic cells, so the binding of annexin V to phosphatidylserine makes this protein a selective and powerful tool for the detection of apoptotic cells [9]. PI reveals the

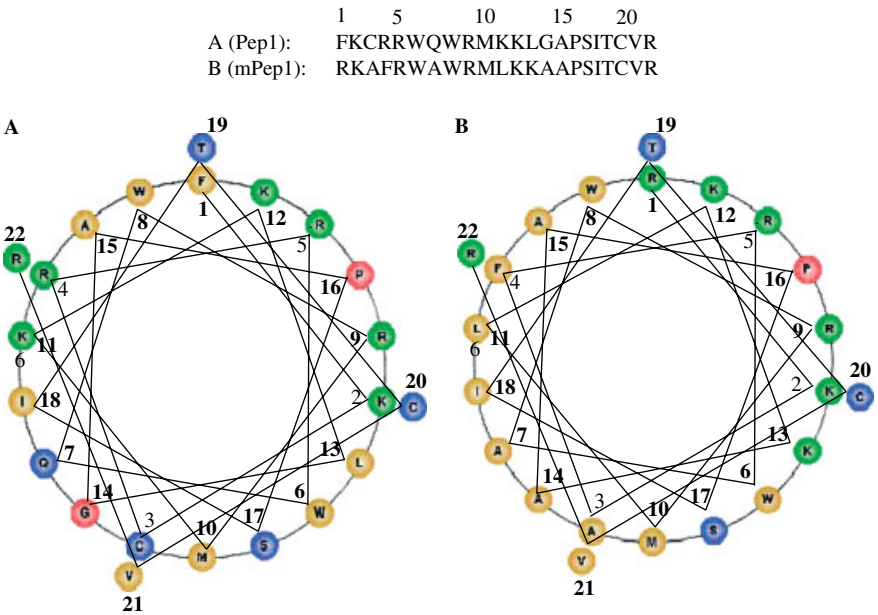

Figure 1 Helical wheel representation of Pep1 and mPep1. (A) Pep1 corresponds to residues 17–38 of Lf-B; (B) mPep1, where residues 3, 7, and 14 of Pep1 were replaced by alanine, and the cationic residues positioned at the minor end (K11, R4) were shuffled to the major end. The helical wheels were created using a Helical Wheel Design Program as described in 'Materials and Methods'. Symbols shown in green are basic amino acids.

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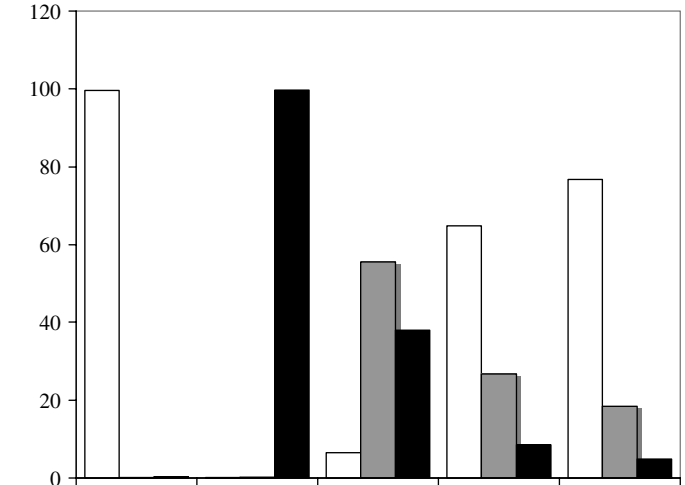
loss of integrity of the plasma membrane, which is specific for necrotic cells. As shown in Figure 3, most of the cells incubated in the presence of 253 μM Pep1 showed characteristics indicative of necrotic cells. However, two-times diluted Pep1, corresponding to 126 μM Pep1, gave a higher percentage of apoptotic cells in comparison to necrotic cells. Further dilution of Pep1 caused a proportional decrease in necrotic cells relative to apoptotic cells, with an inverse increase in the number of living cells. However, all concentrations of mPep1 gave a marked necrotic effect, with little or no apoptosis depending on the dose. For example, an 8 or 16 fold-diluted solution of mPep1, corresponding to 32 and 16 μM Pep1, respectively caused necrosis in 99% of the cells. The mPep1 while further diluted decreased the percentage of necrotic cells, depending on the degree of dilution.

DNA Fragmentation

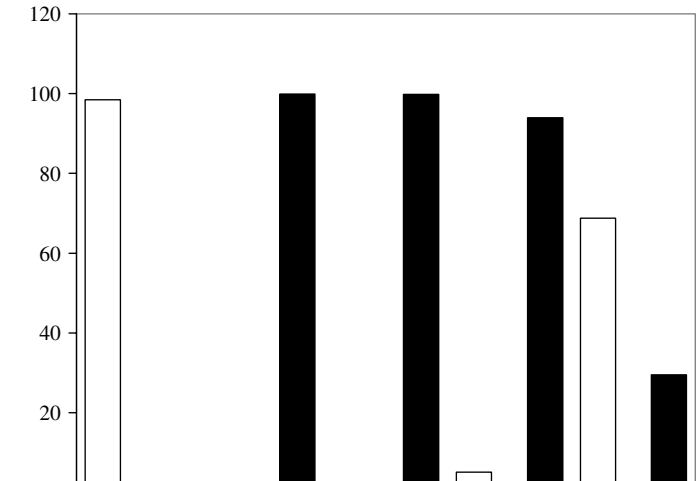
In order to determine the mode of cell death induced by Pep1 or mPep1, DNA was extracted from treated cells and electrophoresed on agarose gels. The results,

A



	Control	253.0	126.5	63.3	31.6
□ Living Cell (%)	99.6	0.1	6.5	64.8	76.8
▒ Apoptotic Cell (%)	0.1	0.2	55.6	26.7	18.4
■ Necrotic Cell (%)	0.3	99.7	37.9	8.5	4.8

B



	Control	31.6	15.8	7.9	4.0
□ Living Cell (%)	98.5	0.05	0.1	5.1	68.8
▒ Apoptotic Cell (%)	0.1	0	0.1	1	1.7
■ Necrotic Cell (%)	1.4	99.95	99.8	93.9	29.5

Figure 3 Two color FACS analysis of HL-60 Cells. Cells were exposed to medium alone (control) or various concentrations of Pep1 (A) or mPep1 (B) for 24 h. After incubation, the cells were collected and incubated with annexin V and PI as described in 'Materials and Methods'. Values are presented as percentage of total cells counted (10 000).

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presented in Figure 4, show that DNA extracted from Pep1-treated cells provided a ladder-like pattern of DNA fragments characteristic of apoptosis, whereas DNA extracted from cells exposed to mPep1 provided a smeared pattern. These results appear to confirm the result obtained in the annexin V/PI assay: Pep1 at an appropriate concentration induces apoptosis, whereas mPep1 almost exclusively induces necrosis.

Activation of Caspase-3 and Release of Cytochrome *c*

While investigating molecular and cellular cascades operating in cell death machinery many previous model studies have shown that ladder-like DNA fragmentation is a consequence of the activation of caspase-3 and release of mitochondrial cytochrome *c* in apoptotic cell death [12]. Thus, cytochrome *c* release and activation of caspase-3 are considered as hallmarks for apoptotic cell death. Our data have shown that Pep1 induced cell viability lost is accompanied by an increased number of apoptotic cells (Figure 3) and ladder-like DNA fragmentation (Figure 4). At this end, we have examined the effect of Pep1 on the activation of caspase-3. As expected, cells exposed to Pep1 exhibited substantial increase in the activation of caspase-3, and the increase was however dependent on the concentration of the peptide. Cells exposed to 126-μM Pep1 showed maximum activation of caspase-3 (Figure 5(A)); however, the activity was decreased proportionally to the degree of dilution. Although mPep1 at a concentration of 4.0–31.6 μM exhibited little effect on the induction of apoptosis, we have examined the effect of mPep1 on the activation caspase-3. Surprisingly, an 8.0-μM mPep1, which caused a 50% loss in cell viability, was found to increase the activation of caspase-3 comprehensively. As expected a higher concentration of mPep did increase the activation, and a complete activation of caspase-3 was observed in the cells exposed to 32 μM mPep1 (Figure 5(B)).

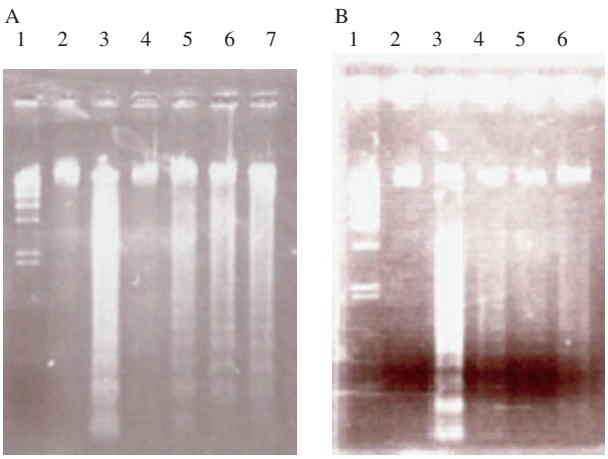

Figure 4 Agarose gel electrophoresis of DNA. DNA was extracted from cells exposed for various times to Pep1 (A) or mPep1 (B). (A) DNA was extracted from cells incubated with 126 μM Pep1 for various times. Lane 1, a standard 200-kbp DNA ladder. Lane 2, cells incubated with medium alone. Lane 3, cells incubated with actinomycin D. Lanes 4–7 contain DNA from cells incubated with Pep1 for 0, 6, 12, and 24 h, respectively. (B) DNA was extracted from cells incubated with 32 μM mPep1 for various times. Lanes 4–6 contain DNA from cells incubated with mPep1 for 6, 12, and 24 h. Experimental conditions for each of 1–3 lanes are the same with those in (A) (Pep1).

Many previous studies have shown that caspase-3 activation is linked with the release of cytochrome *c* from mitochondria into cytosol. To observe, if there is any link in the activation of caspase-3 to its upstream activator molecules such as cytochrome *c*, we have examined the effect of Pep1 and mPep1 on cytochrome *c* release from the mitochondrial fraction. As expected, in accordance with the occurrence of annexin V positive cells, DNA fragmentation, and caspase-3 activation, Pep1 at a concentration of 126 μM induces cytochrome *c* release into cytosol (Figure 6) in a time dependent manner. Herein, we have concluded that Pep1 induced cytochrome *c* released could be linked to classic mechanism of mitochondrial dependent apoptotic cell death pathway. For investigating the minimal consequences of mPep1 on the release of cytochrome *c*, we used a low dose, 4 μM, which exhibits the least effect in inducing cell death as determined by MTS and annexin V/PI assays. We have observed that mPep1 at such a low concentration induces cytochrome *c* release into the cytosol (Figure 6). Despite the release of cytochrome *c* at such a low dose and also activation of caspase-3 when cells were exposed to another lower dose (8 μM), mPep1 induced necrosis rather than apoptosis as determined by the annexin V/PI assay. This observation was however in contrary to many studies wherein necrotic cell death has been described as a passive process of cell death. Our observation proposed that necrotic cell death can be an active process requiring the activation of cytochrome *c* release into cytosol and activation of caspase-3.

The antitumor activity of milk proteins, particularly Lf-B and peptides derived from it, has been recently investigated [13,14]. Peptides derived from the *N*-terminal region of Lf-B, comprising an α-helical structure between 29 and 31 residues long were found to have the highest lytic activity toward prokaryotic as well as transformed eukaryotic cells [15]. Thus, the molecular basis of the cytotoxicity of Lfcin-B or Pep1 can be attributed to their highly basic, amphipathic α-helical structure that allows the peptide to interact with the negatively charged plasma membrane of the tumor cells. Structural parameters related to the location of cationic residues in peptides derived from the *N*-terminal end of Lf-B could be used to improve its therapeutic efficacy against tumor cells. For this

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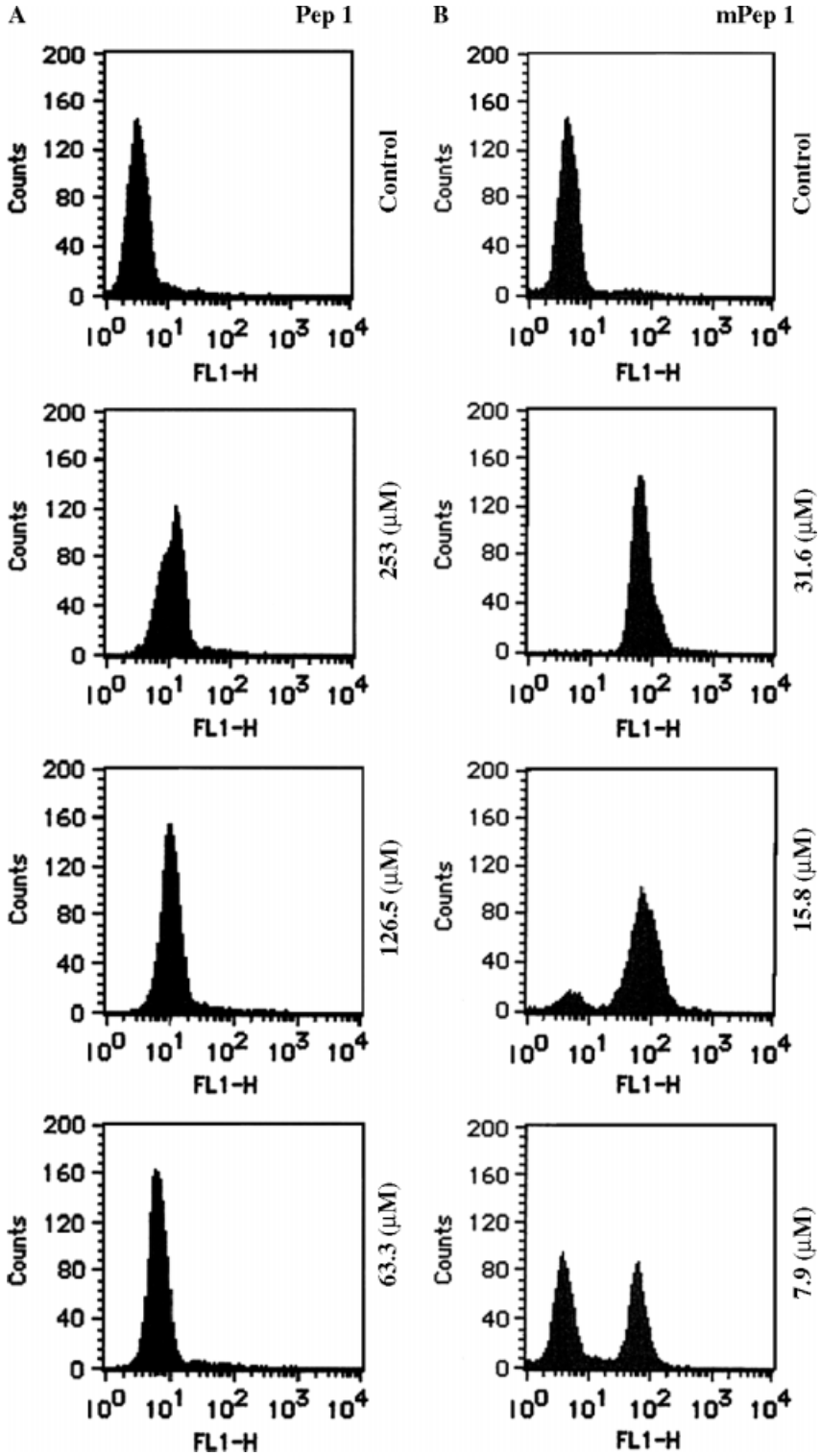

Figure 5 FACS analysis of caspase-3 activation in HL-60 Cells. Caspase-3 activation was measured by flow cytometry as described in 'Materials and Methods'. (A) Cells were incubated for 12 h with medium alone (control) or, 126, 63, or 32 μM Pep1, respectively. (B) Cells were incubated for 12 h with medium alone (control) or 32, 16, or 8 μM mPep1, respectively.

purpose, Yang *et al.* investigated the principal characteristics of the helical structures of these peptides and showed that peptides with a cluster of cationic residues in the helical region exhibited enhanced lytic activity toward transformed cells [7]. However, the study did not identify the type (apoptosis or necrosis) or mode of cell death. We therefore investigated the mode of cell death induced by Pep1 and mPep1. Pep1 caused phosphatidylserine to migrate from the inner to the outer leaflet of the plasma membrane, and gave a

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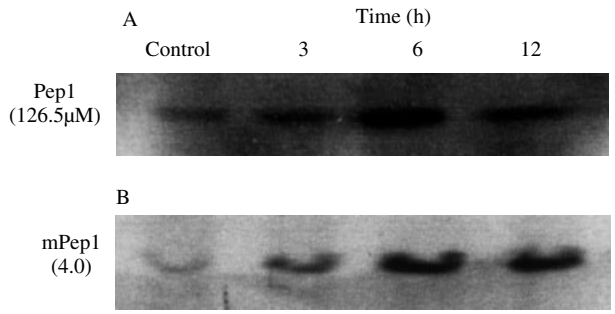

Figure 6 Western Blot Analysis for cytosolic cytochrome *c* in HL-60 Cells. Cells were incubated with 126 μM Pep1 (A) or 4 μM mPep1 (B) for 3, 6, and 12 h. The control is the sample extracted from cells not exposed to Pep1 or mPep1. Cytosolic extract was separated on 12% SDS-PAGE and the proteins were electro-blotted onto a PVDF membrane. The blot specific to cytochrome *c* was identified using a specific cytochrome *c* antibody, and then visualized.

ladder-like DNA fragmentation pattern. These findings suggest that an appropriate concentration Pep1 can activate apoptosis-inducing signal pathways in HL-60 cells, whereas mPep1 may severely damage the membrane structure of cells. Apoptotic cells are characterized by several unique features including cell shrinkage, chromatin condensation, expression of phosphatidylserine on the outer cell surface [15], and ladder-like fragmentation of nuclear DNA. By contrast, in necrotic death, the nucleus is relatively preserved in the early stages, while cytoplasmic organelles and the cell membrane are disrupted. However, recent studies suggest that necrosis can also be due to programed cell death, and that it shares several biochemical features with classical apoptosis [13,16]. In this study, we demonstrated that the massive necrotic cell death induced by mPep1 caused the release of mitochondrial cytochrome *c* and activation of caspase-3. Thus, caspase-3 activation in mPep1-induced necrotic cells confirms earlier reports of caspase-3 involvement in necrosis. However, it is not clear whether the activation of caspase-3 or release of cytochrome *c* into the cytosol is functional here, since we did not observe any increase in the viability of cells preincubated with a general inhibitor of caspases prior to mPep1 exposure (data not shown). Niquet *et al.* showed that inhibition of protein synthesis could block cytochrome *c* release and caspase-3 activation during apoptosis, but had no effect on 'programed necrosis' [17]. Therefore, massive caspase-3 activation or influx of cytochrome *c* from the mitochondria to the cytosol may or may not be due to necrotic cell death induced by mPep1. Additional experiments will be necessary to clarify this point.

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