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The cytotoxic effects of the anti-bacterial peptides on leukocytes

Sergiu-Bogdan Catrina,[a,b] Essam Refai[a,b] and Mats Andersson[a,c,d]*

Antimicrobial peptides are small molecular weight proteins with a large antibacterial spectrum. They can reach high local concentrations in tissues with active inflammation, being largely produced by immunocompetent cells. However, their effect on eukaryotic cells is still unclear. We have, therefore, studied three structurally different antimicrobial peptides (cecropin P1, PR-39 and NK-lysin) for their cytotoxic effects on blood mononuclear cells. None of the antimicrobial peptides tested exhibited significant cytotoxic effect on resting lymphocytes isolated either from peripheral blood or from the spleen with the exception of high concentrations (ten times higher than IC100 for *Escherichia coli*) of NK-lysin. Activated lymphocytes were, however, more sensitive to the cytotoxic effect of the antimicrobial peptides. Both activated T-cells and B-cells were dose dependent sensitive to NK-lysin while only activated B-cells but not activated T-cells were sensitive to PR-39. Cecropin did not exhibit any cytotoxic effect on activated lymphocytes either. By using several cell lines (3B6, K562, U932 and EL-4) we were able to show that NK-lysin has a broad necrotic effect while PR-39 has a cell specific apoptotic effect dependent on the specifically cellular uptake. In conclusion we show here that antimicrobial peptides are not cytotoxic for the resting eukaryotic cells but can be cytotoxic on activated immune cells through distinct mechanisms of cell death. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: innate immunity; cell death; NK-lysin; PR-39; cecropin P1

Introduction

Antibiotic peptides are common in the animal kingdom and represent the first line of defense against pathogens. Most of these peptides are active against a broad spectrum of bacterial and fungal strains while some peptides exhibit antiviral activity, as well [1,2]. The peptides differ in sequence and folding but share the properties of having a high basic net charge and often an amphiphilic structure. The main mechanism of action of the antibacterial peptides involves cellular permeabilisation even though the exact mechanism of membrane disruption is not completely understood (recently reviewed by Zhang and Falla [3]). It is, however, well established that some of the antibacterial peptides do not cause membrane permeabilization but translocate across the membrane where they target a different essential cellular processes to induce cell death [4]. The mammalian antibacterial peptides exhibit some toxicity to the transformed cells above their antimicrobial activity [5–10] but their effect on normal eukaryotic cells is largely unknown. The cytoxic mechanisms are still not defined, although DNA breaks and apoptosis-inducing events in the target cell have been observed for certain peptides [11–16].

NK-lysin, PR-39 and cecropin P1 are three natural occurring antibacterial peptides structurally different. NK-lysin and its human alike, granulysin, are 8 kDa disulfide constrained peptides co-stored in the cytolytic granules with perforin with which contributes to the lysis of bacteria and infected cells [10,17,18]. NK-lysin is active against bacteria (including *Mycobacterium tuberculosis*), fungi and tumour cells [10,19,20]. It interacts with membranes and lyses lipid vesicles but the activity does not involve persistent pore formation [21]. PR-39 is a proline–arginine rich antibacterial peptide [22] produced and stored in circulating granulocytes [23,24]. It kills bacteria by a non-lytic mechanism through inhibition of DNA and protein synthesis [25]. PR-39 can cross mammalian cell membranes and interacts with Scr homology 3 (SH3) domains of $p47^{phox}$, regulating anti-inflammatory events [26]. It interacts also with p130cas, a protein involved in the integrin mediated signal pathways [27]. The peptide is found at sites with active inflammation and may play important role in wound healing through the induction of syndecan expression [23,28] and through recruitment of neuthrophils [27]. PR-39 also inhibits the ubiquitin-protesome pathway-mediated degradation of essential intracellular signal proteins as hypoxia-inducible factor-1α (HIF-1α) [29] or IκBα [30].

High local concentration of antibacterial peptides at inflammatory sites may in addition to its sterilizing activity also negatively affect bystander cells. The present study investigates the potential cytotoxic activity of cecropin P1, PR-39 and NK-lysin against primary leukocytes. We have noted that cecropin P1 that has the broadest antibacterial spectra, has the lowest cytolytic activity. Both NK-lysin and PR-39 are cytoxic to *in vitro* activated lymphocytes but just high concentrations of NK-lysin are cytotoxic against

* *Correspondence to: Mats Andersson, Department of Microbiology, Tumor and Cell Biology, Karolinska institutet, Stockholm, Sweden. E-mail: mats.andersson.2@ki.se*

a *Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden*

b *Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden*

c *Department of Microbiology, Tumor and Cell Biology, Karolinska institutet, Stockholm, Sweden*

d *Department Immunologie, Institute Pasteur, Paris, France*

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resting lymphocytes. The mechanism of cell death was further studied in different cell lines having in mind the potential use for these peptides in oncology.

Materials and Methods

Materials

NK-lysin and PR-39 were isolated from pig intestine and cecropin P1 was synthesized as described [10,22,31]. RPMI was from Sigma (St. Louis, MO). Fetal calf serum (FCS) and antibiotics were from GIBCO (Paisley, Scotland). The cyto96 cytotoxic kit was from Promega (Madison, WI), alamar blue from Biosource, Alexa 594 from Molecular Probes (Eugene, OR), Annexin V/Propidium Iodide apoptosis kit (R & D System, Minneapolis, MN).

Target Cells

Leukocytes

Leukocytes used were from balb/c mouse.

Peripheral blood mononuclear cells (PBMC)

Blood was collected and mononuclear cells were partially purified by ficoll (density 1.077, Seromed) sedimentation. The ficoll layer fraction was collected, washed once in PBS and used for cytolytic assays.

Spleen T-cells

Cells were prepared from spleen and then incubated with anti-Ig coated Petri dishes in order to reduce the number of B-cells. After two incubations the cells in suspension were collected, adjusted to 0.5×10^6 cell/ml and plated in 24 well plates. The cells were then stimulated with concanavalin A (5 μg/ml) and IL-2 (10 U/ml) for seven days. By this method the percentage of B-cells (Ig positive) was 30% before stimulation with concavalin A and IL-2, 30% at day 2 and 0% at day 5–6 while T-cells (CD3 positive) were 40% at day 0, 55% at day 2 and 90% at day 5–6 as checked by FACS (on gated lymphocyte population). The ratio of $CD4^+/CD8^+$ changed during cultivation from 2.6/1 to 1/1.75 indicating a preferential stimulation of $CD8^+$ cells.

Spleen B-cells

After collection of the spleen cells, the T-cells were lysed by incubation for 45 min at 37 °C with supernatant (1 : 5) from JIJ hybridoma cells (rat IgM anti-mouse Thy 1.2) together with guinea-pig serum as a source of complement (1 : 10). B-cells were collected afterwards by ficoll centrifugation and finally plated (0.5×10^6 cell/ml) and stimulated with LPS (*Salmonella enterica* serovar typhimurium, 10 μg/ml).

Cell lines

Cell lines used were 3B6 (721/84.5 Eppstein-Barr virus (EBV)-infected lymphoblastoid B-cell line) [32], K562 (human erythroleukemia cell line), U937 (human histocytic lymphoma cell line) and EL-4 (mouse lymphoma cells). They were maintained suspended in RPMI-1640 culture medium, supplemented with 10% heat-inactivated FCS, glutamine and 100 IU/ml penicillin and streptomycin, in 5% CO_2 atmosphere. For experiments, a density of 2×10^5 cells/ml in RPMI-1640, 2% FCS was used after counting of the viable cells with trypan blue.

Cytotoxic Activity of the Peptides

Cytotoxic activity of the peptides was assessed by different methods, as appropriate, after incubation for 1–8 h (routinely 4 h) of the cells (100 μl of cell suspension) with 10–20 μl of either peptide sample diluted in medium (test) or medium only (control). The tests were performed in 96-wells plates in triplicate.

Lactate dehydrogenase (LDH) activity

LDH activity released in the medium was assessed using the cyto96 cytotoxic kit according to the manufactures instructions.

Alamar blue

This assay was used for evaluating the cell viability. Briefly 10 μl *alamar blue diluted in* 50 μl fresh RPMI-1640 with 2% FCS was added to each well and the percentage of inhibition of cell growth was calculated after 10 h incubation according to the manufacture's instructions.

^{51}Cr release assay

It was performed as previously described [33]. Briefly the cells (400 μl) were mixed with 100 μl of $Na_2-{}^{51}CrO_4$ (2 mCi/ml) and incubated at 37 °C for 1 h. Labelled cells were washed three times and then resuspended in medium supplemented with FCS (20 000 cells/well). The cells were then treated with antibacterial peptides (as indicated) or control and at the specified time points centrifuged for 4 min at $100 \times g$ and the supernatant collected for radioactive assay. Results are expressed as percentage of control, untreated cells at the specified time point.

Propidium iodide (PI)

PI retention in peripherial lymphocytes was evaluated by flow cytometry (FACScan, Becton-Dickinson, Electronic lab, Mountain View, CA, USA). *PBMC* (1×10^5) after incubation with either antibacterial peptides or control were incubated with PI for 15 min at a final concentration of 5 mg/ml. The percentage dead cells were estimated as (PI-sample) – (PI-control) in a gate not including debris. Lymphocytes were separately evaluated by staining with anti-mouse CD3-FITC and anti-mouse B220-FITC and gated. The amount of dead lymphocytes was estimated indirectly as the disappearance of cells in this gate.

Apoptosis *Versus* Necrosis of K562 Cells

The K562 cells (10^6 cells/ml) were seeded in 24-well plates (450 μl together with 50 μl test solution). Apoptosis was evaluated by analysing phosphatidylserine exposure by annexin V-FITC staining as previously described [34]. Cells were incubated for different times with NK-lysin or PR-39 or control and then incubated with a mixture of fluorescein-conjugated annexin V and PI at room temperature for 15 min in the dark and thereafter immediately analysed by FACS. Compensation was performed with samples stained only with annexin V or propidium. *Living cells* were defined as those negatively stained for both PI and annexin V (lower left quadrant). *Apoptotic* cells were defined as those staining positive for annexin V and negative for PI (lower right quadrant); *necrotic cells* were defined as those staining only with PI and *late apoptotic or late necrotic cells* were defined as those staining positively for both. Values are expressed as percent of cells gated in a certain quadrant.

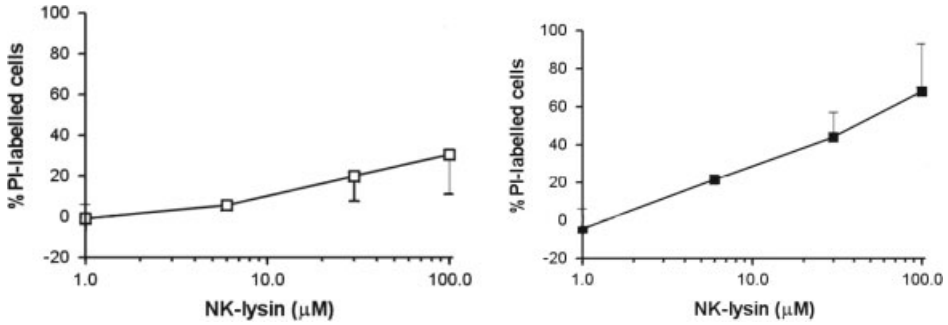

Figure 1. NK-lysin can have cytolytic effects on white blood cells especially on lymphocytes. PI-labeled cells counted in whole population (A) and disappearance of cells in a lymphocyte gate defined after stained the whole population separately with anti-CD3-FITC and anti B220-FITC (B). Data is presented as the mean of two separate experiments in duplicate.

Cellular Uptake of Pr-39

PR-39 was fluorescent labeled with Alexa 594™ (Invitrogen, Molecular Probes, Eugene, OR) (according to manufactures instructions) and purified by RP-HPLC. The fluorescent PR-39 preserved high antibacterial activity against *Escherichia coli* (data not shown). Labeled PR-39 (30 μg/ml) was incubated with either 3B6 or U937 for 2 h and then analysed under the fluorescent microscope.

Results

The Antibacterial Peptides Have Different Cytotoxic Effects on Resting *Versus* Active White Blood Cells

We first studied the cytotoxic effects of the antibacterial peptides (NK-lysin, PR-39, cecropin) on PBMC. While PR-39 and cecropin P1 had practically no cytotoxic effect on PBMC, NK-lysin exhibited cell toxicity in a dose dependent manner as shown by retention of PI (Figure 1). Significant cytotoxic effects were noted, however, at high concentrations of NK lysin i.e. 30% of PBMC were killed after exposure to 100 μM NK lysine which is a concentration ten times higher than IC100 for *E. coli* [10]. Lymphocytes were more sensitive to NK lysin cytolytic effects then the whole PBMC population, as 50% of the lymphocytes accumulated PI after exposure to 30 μM NK-lysine as evaluated from the disappearance of cells in the B-($B220^+$) and T-cells ($CD3^+$) positive gates.

Having in mind that the potential highest concentration of the antibiotic peptides is reached at the sites of inflammation, where mainly activated lymphocytes are present we have further investigated the effect of the antibacterial peptides on activated B- and T-lymphocytes by ^{51}Cr release (Figure 2). In perfect agreement with the data obtained from the gated lymphocytes in PBMC, just NK-lysin showed low but significant lysis of both resting B- and T-cells (Figure 2(B) and (C) day 0). PR-39 lysed only resting T-cells and not B-cells (Figure 2(B) and (C) day 0) which is consistent with the unsignificant effect of PR-39 on PBMCs lysis since the majority of PBMCs cells are B-cells (about 80% according to FACS analyses). Again cecropin P1, tested up to 50 μg/ml, had no toxic effect on any resting lymphocyte subtype.

Both activated B- and T-cells were more sensitive then the resting cells to the cytotoxic effects of the antibacterial peptides. NK-lysin killed twice more activated B- and T-cells than resting lymphocytes (Figure 2(B) and (C) days 2–5) exactly as PR-39 which remained cytotoxic only for activated T-cell but not for activated B-cell.

The cytotoxic effects of both NK-lysin and PR-39 are independent of new protein synthesis as far as exposure of the activated T-cells to cycloheximide for 4 h prior to the incubation with the antibacterial peptides did not influenced the cytotoxic effects (data not shown).

The Antibacterial Peptides Have Different Cytotoxic Mechanisms

The cytotoxic mechanisms of the three prototype antibiotic peptides were further studied in several cell lines (Figure 3). As for the primary white blood cells, the highest cytolytic effect was exhibited by NK-lysin in all cells tested. In perfect agreement with the lack of cellular toxicity observed in primary cells cecropin P1 had no cytolytic effects on any of the cell lines tested. PR-39 exhibited a selective cytotoxic effect on cell lines as well as on primary lymphocytes being most cytotoxic on 3B6 cells intermediary towards K562 and not at all on U937 cells (Figure 3(A)). The cytotoxic mechanisms of the two antibacterial peptides (NK-lysin and PR-39) that showed cytotoxicity both in primary cells and in cell lines were further studied by longitudinally evaluation of their effects on cellular metabolic activity (by alamar blue staining) and integrity of the cellular membrane (LDH release) in the cell lines sensitive (K562 and 3B6) (Figure 3(B–D)) While LDH release and inhibition of the metabolic activity coincide during incubation with NK-lysin in both cell lines tested, the metabolic inhibition clearly precedes the release of LDH for PR-39.

The cytotoxic effect of the antibacterial peptides is specifically dependent on the cellular uptake. In order to further characterize the mechanisms of action of the antibacterial peptides we took advantage of the difference in cytotoxic effect of PR-39 for different cell lines. In perfect agreement with the effect observed on both metabolic cellular activity and membrane integrity, labeled PR-39 showed distinct staining just in 3B6 cells and diffuse, unspecific staining in U937 cells (Figure 4). This suggest that specific cellular uptake of the peptide is needed for PR-39 cytotoxic activity.

Apoptosis *Versus* Necrosis

Taking in account the different effect of NK-lysin and PR-39 on cellular metabolism and cellular membrane integrity we have further analysed the mechanism of cell killing. We have chosen K562 cells, for this purpose, because the cells were susceptible to both NK-lysin and PR-39. Cells were incubated with 50 μg/ml NK-lysin or 100 μg/ml PR-39 (representing about 25–60% lysed cells after 4 h incubation according to the cytolytic assay, Figure 3). NK-lysin did not induce any significant apoptosis (annexin V

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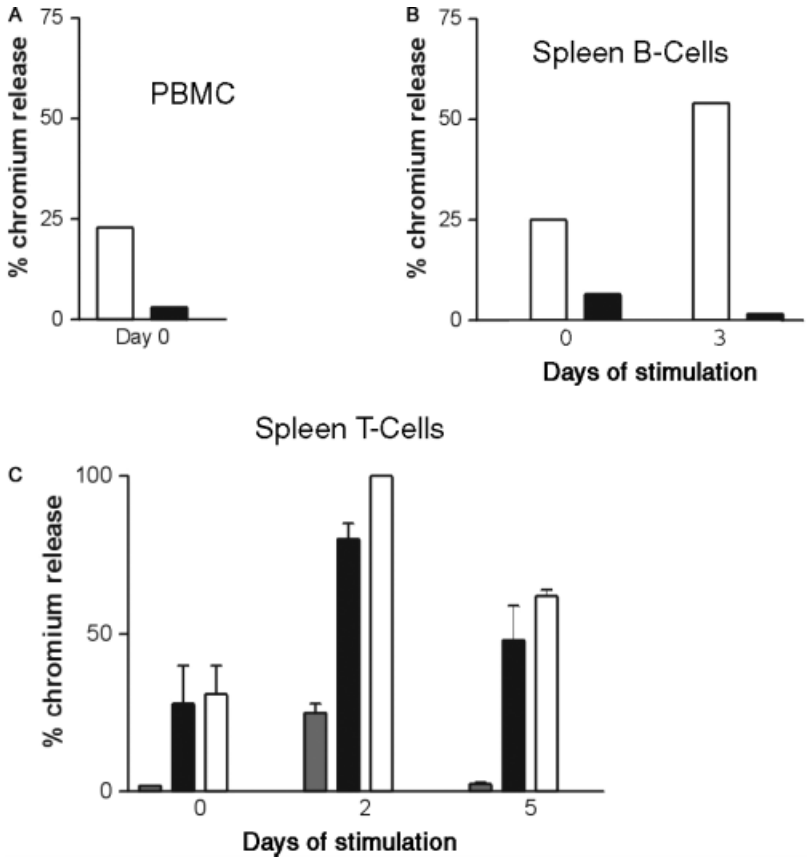

Figure 2. Lysis of lymphocytes by NK-lysin, PR-39 or cecropin P1 (50 μg/ml). Cells were collected from blood or prepared from spleen and incubated for 4 h at 37 °C with NK-lysin (open), PR-39 (solid) or cecropin P1 (shaded). Lysis of naive cells (A), B-cells stimulated with LPS (B) and T-cells stimulated with concanavalin A and IL2 (C).

positive and PI negative) (Figure 5). PI staining was about 20%, which is comparable to the cytolytic data (Figure 3). Prolonged incubation (up to 24 h) resulted in a higher uptake of PI but no additional phosphatidylserine staining. However incubation with PR-39 showed a pronounced Annexin V staining that increased by time. About 32% of the cells were Annexin V positive after 4 h showing that PR-39 is activating apoptosis (Figure 5).

Discussion

Animal peptide antibiotics represent the first line of defense against invading pathogens. On a structural basis, antibacterial peptides can be divided into three groups, amphipatic alpha helical peptides (e.g. cecropin P1), compact disulphide locked peptides (e.g. NK-lysin) and others, some with a high proportion of a specific amino acid (e.g. the proline–arginine rich PR-39). Most of the peptides are active at low micromolar concentrations against a broad range of bacteria [1]. PR-39 is present in neutrophils, assisting in the lysis of engulfed bacteria and NK-lysin in cytolytic cells where it is involved in the granule dependent delivery of cytotoxic molecules to the target cells. These peptides that are released during an inflammatory process may potentially affect bystander cells other than the target cell. We studied the cytotoxicity of NK-lysin, PR-39 and cecropin P1 on normal cells in quiescent as well as in stimulated state. It has been suggested that these peptides have evolved for intracellular functions and thus are not a major threat to the host. It is partially confirmed by our results where none of the three antibacterial peptides tested, representing different prototype peptide, has an important cytolytic effect on resting fresh lymphocytes even in concentrations ten times higher than that required for antibacterial activity. However, activated lymphocytes are sensitive to antibacterial peptides and could be damaged. PR-39 exhibits a selective cytolytic activity against T-cells but not against B-cells, but NK-lysin, on the other hand, is not selective and lyses all the cells tested. Thus, high levels of antibacterial peptides released in inflammatory sites can contribute to cell death in the infected tissues.

We further looked on the mechanisms of cytotoxicity of three antibacterial peptides by using different cell lines: human EBV infected B-cell line (3B6), human histocytic lymphoma cell line (U937) and a human erythroleukemia cell line (K562). Cecropin P1 has no cytotoxic effect on any cell tested. On the other hand, NK-lysin and PR-39 have clear cytotoxic effects. NK-lysin was a very potent cytotoxic factor against all the cell lines tested showing a dose-dependent effect. PR-39 was cytotoxic, practically, against 3B6 and K562 but not against U937. The difference in sensitivity of the tumour cells for PR-39 could be explained by the biding studies. We showed a specific uptake of the peptide by 3B6 but not by U937 (Figure 4) suggesting that its effect requires specific interactions. Many arginine-rich peptides efficiently translocate through cell membranes by common mechanisms [35] where cell specificity might depend on the degree of surface proteoglycans [36].

Once internalized PR-39 binds to SH3-containing proteins [26,27] that could contribute to its pro-apoptotic effect [37].

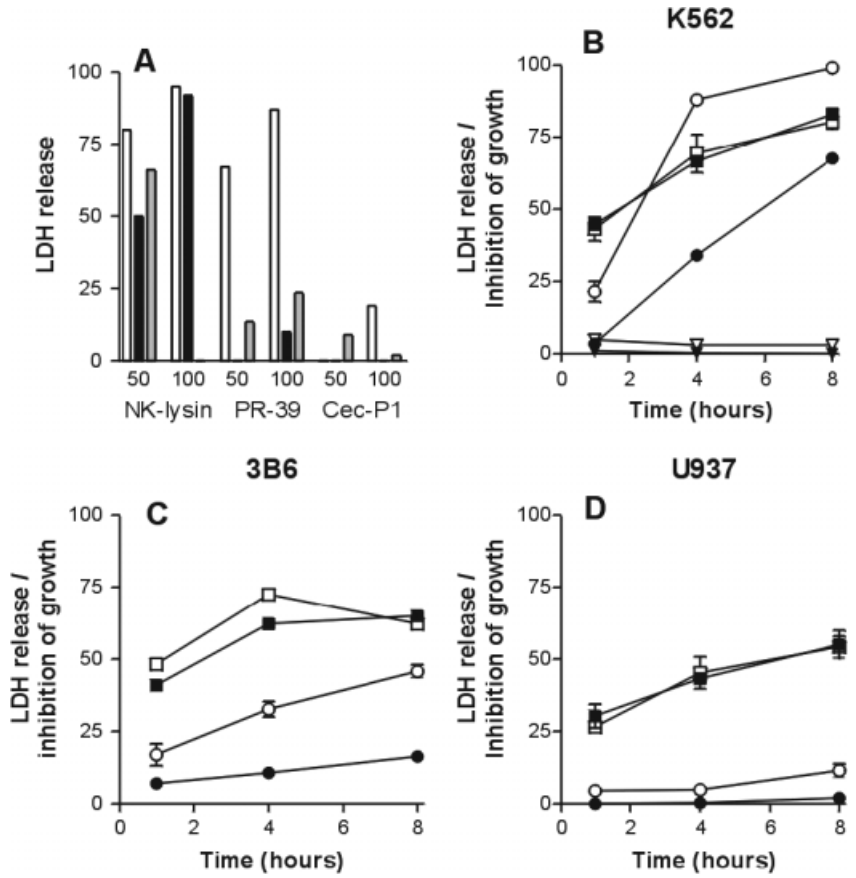

Figure 3. Lysis of tumour cell lines by antibiotic peptides. (A), 3B6 (open), U937 (solid) or K562 (shaded) cells were incubated in the presence of NK-lysin, PR-39 or Cecropin-P1 for 4 h. Peptide concentrations are in μg/ml. Cell lysis was determined by analysing the LDH activity released in the supernatant. (B) K562 cells were incubated with 50 μg/ml NK-lysin (■, □), 100 μg/ml PR-39 (●, ○) or cecropin P1 (▽, ▼) (C) 3B6 cells were incubated with 25 μg/ml NK-lysin (■, □), 25 μg/μl PR-39 (●, ○). (D) U937 cells were incubated with 25 μg/ml NK-lysin (■, □), 25 μg/μl PR-39 (●, ○). Both LDH release (filled symbols) and inhibition of cell growth (open symbols) were determined. Data represent the mean of two experiments performed in triplicate.

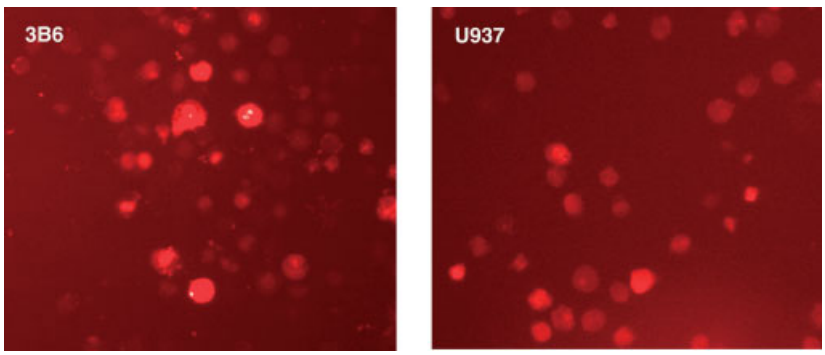

Figure 4. Cellular uptake of PR-39: Incubation with fluorescent-labeled PR-39 for 2 h shows distinct positive staining in 3B6 cells (white spots) and diffuse, unspecific staining in U937 cells.

Moreover, PR-39 interferes with the ubiquitin–protesome mediated degradation of specific proteins [29,30] However, PR-39 is not able to block the overall protein degradation that could possibly be linked to cell death (N.P. Dantuma, unpublished). The proaptotic effect of PR-39 is highly cell specific as far as opposite effects were described in Hela cells, cardiomyocytes or endothelial cells [38–40].

The possibility that antibiotic peptides are toxic by inducing apoptosis and DNA fragmentation has been suggested in studies with granulysin [14], BMAPs [15], mastoparan [41] and cecropin–melittin [42]. The pathways activated by these peptides that induce apoptosis have not been clearly identified but at least two peptides, granulysin [16] and BMAP [15], act by disturbing the mitochondria. Apart from granulysin, shown to act through both a caspase dependent and caspase independent pathway, other peptides activities do not respond to caspase inhibitor. Inhibitors to caspase 9, 2 and 3 did not notably reduce the effect of PR-39 (data not shown). The cytolytic effect of NK-lysin is mainly necrotic as shown by early retention of PI *versus* annexin but does not involve persistent pores [21] or increase of Ca^{++} influx [43]. The

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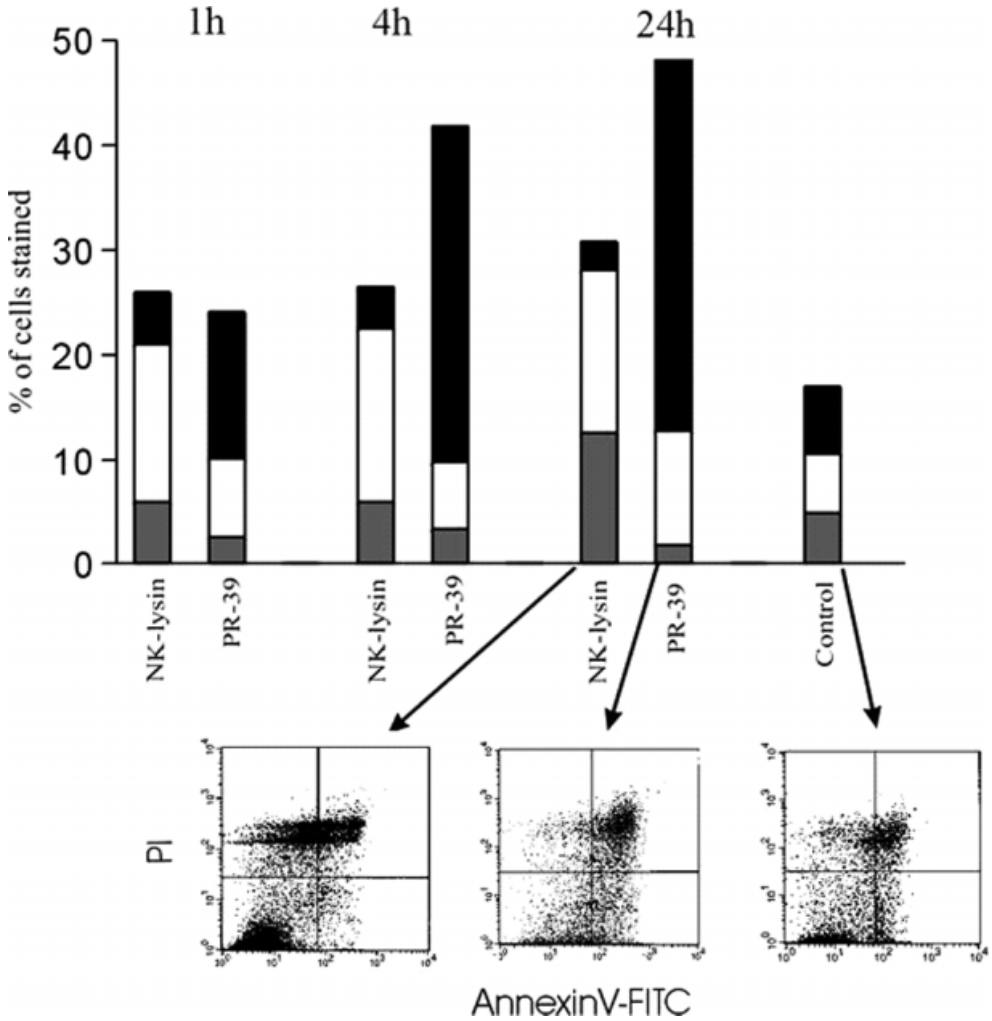

Figure 5. Annexin V and PI staining of K562 cells after incubation with NK- lysin or PR-39. Cells were incubated for different times with 50 μg/ml NK-lysin or 100 μg/ml PR-39 and thereafter washed and stained simultaneous with annexin V-FITC and PI. Cells were analysed by FACS and the percentage of cells stained in the different quadrants summarized. PI positive (open), annexin V positive (solid) and PI plus annexin V positive (shaded). Inset; representative FACS presentation after 24 h incubation of cells ± peptides.

other two peptides that are homologous to NK-lysin have also cytolytic effects by different mechanisms. While amoebapores induce-like NK-lysin, mainly necrosis and do not require Ca^{++} for this [44] granulysin acts apparently through an apoptotic pathway [14] although an alternative necrotic mechanism can occur at high peptide concentration [13]. It is noteworthy to mention that central synthetic fragments of granulysin maintain the apoptotic properties of full-length peptide [14] while the corresponding fragments of NK-lysin are necrotic (data not shown). Thus it appears to be a fundamental difference in the mode of action against mammalian cells between these homologues peptides. An increase of the intracellular levels of Ca^{++}, associated with apoptosis [45] is observed after treatment with BMAP, cecropin–mellitin and mastoparan [12,41,42]. It is possible that peptides affecting the Ca^{++} levels (mastoparan, BMAP, ceropin-mellitin, PR-39) are capable of inducing apoptosis while others (NK-lysin, amoebapores) are mainly necrotic.

Our observation of a potential cytotoxic and pro-apoptotic effect on activated T-cells of PR-39 could be of importance thinking of the development of PR-39 and related compounds as inducers of angiogensis [29].

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