Library Authorization

In presenting this thesis in partial fulfillment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Yee-Lar Elaine Lau  
Name of Author (please print)  
18/06/2004  
Date (dd/mm/yyyy)

Title of Thesis: Characterizing the interactions between the host defence peptide, LL-37, and lung epithelial cells

Degree: Masters of Science  
Year: 2004

Department of Microbiology and Immunology

The University of British Columbia

Vancouver, BC  Canada
ABSTRACT

LL-37 is a human cationic host defense peptide that is a component of innate immunity. In addition to its modest antimicrobial activity, LL-37 affects the behavior of effector cells involved in the innate immune response, including modification of transcriptional responses. However, its mode of interaction with eukaryotic cells remains unclear. In this research study, the interaction of LL-37 with epithelial cells was characterized in tissue culture by using biotinylated LL-37 (LL-37B) and confocal microscopy. LL-37 was actively taken up into A549 human epithelial cells and eventually localized to the perinuclear region. Specific inhibitors were used to demonstrate that the uptake process was not mediated by actin but required elements normally involved in endocytosis, and that trafficking to the perinuclear region was dependent upon microtubules. Using Scatchard analysis, it was revealed that A549 epithelial cells have two receptors for LL-37B, with high and low affinity for LL-37. Further studies, consistent with other publications, that, at higher concentrations, LL-37 demonstrated some cytotoxicity. Therefore the cytotoxic nature of LL-37 was examined in the presence of different sources of serum. LL-37 was cytotoxic in the presence of 10% fetal bovine serum but not 10% human serum and that cytotoxicity led to apoptosis, as assessed by TUNEL assay and Western blot for pro-caspase-3. High density lipoproteins present in human serum, but not the major constituent in HDL, apolipoprotein A-1, inhibited LL-37-induced cytotoxicity. The localization of LL-37B differed according to the type of serum present during incubation. These results suggest that LL-37 interacts directly with epithelial cells and further our understanding of its role in modulating the innate immune response.
# TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... ii

TABLE OF CONTENTS ........................................................................................................ iii

LIST OF FIGURES .............................................................................................................. v

LIST OF ABBREVIATIONS .................................................................................................... vii

ACKNOWLEDGMENT ........................................................................................................... ix

1. INTRODUCTION ........................................................................................................... 1
   1.1 Properties and function of cationic antimicrobial peptides ........................................ 1
   1.2 Cathelicidins .................................................................................................................. 3
   1.3 Receptor-mediated endocytosis and cellular transport .................................................. 6
   1.4 Cytotoxicity and cell death .......................................................................................... 9
   1.5 Aims of this study ........................................................................................................ 11

2. MATERIALS AND METHODS ...................................................................................... 12
   2.1 Peptide synthesis .......................................................................................................... 12
   2.2 Biotinylation of LL-37 ................................................................................................. 12
   2.3 Cell line maintenance ................................................................................................... 12
   2.4 Liposome preparation ................................................................................................... 13
   2.5 Circular dichroism spectrometry ................................................................................ 13
   2.6 LDH assay for cytotoxicity ......................................................................................... 14
   2.7 Cytokine production in A549 epithelial cells ............................................................... 14
   2.8 RNA isolation ............................................................................................................. 15
   2.9 Microarrays ................................................................................................................ 15
   2.10 Immunofluorescence ................................................................................................. 16
   2.11 Inhibition studies with immunofluorescence ............................................................. 17
   2.12 Co-localization studies with endocytic markers ....................................................... 18
   2.13 Binding assays .......................................................................................................... 18
   2.14 Antibodies ................................................................................................................ 19
   2.15 Real-time quantitative RT-PCR ............................................................................... 19
   2.16 TUNEL assay ............................................................................................................ 20
   2.17 Western blot analysis ............................................................................................... 21
   2.18 HDL/Apolipoprotein A-1 isolation from whole blood ............................................... 21
   2.19 Statistical analysis .................................................................................................... 22
3. RESULTS .......................................................................................................................... 23

3.1 Interaction and cellular localization of the host defence peptide, LL-37, in lung epithelial cells .......................................................................................................................... 23

3.1.1 Introduction .................................................................................................................. 23
3.1.2 Biotinylated LL-37 and LL-37 have similar structural and biological properties .... 23
3.1.3 Uptake of LL-37B into cells is an active process resulting in localization to the perinuclear region ................................................................................................................... 25
3.1.4 Uptake of LL-37B incorporates elements of endocytosis, and LL-37 trafficking is mediated by microtubules ...................................................................................................... 29
3.1.5 Uptake of LL-37B is not mediated by G, protein-coupled receptors .................... 34
3.1.6 LL-37 induced IL-8 expression is inhibited by brefeldin A .................................. 38
3.1.7 LL-37B is bound by two receptors and the low affinity receptor appears to be FPRL-1 ................................................................................................................................. 42
3.1.8 Summary ..................................................................................................................... 42

3.2 Activities of LL-37 in lung epithelia ............................................................................. 45

3.2.1 Introduction .................................................................................................................. 45
3.2.2 The cytotoxicity of LL-37 on A549 lung epithelial cells is serum dependent ....... 45
3.2.3 Localization of LL-37B in lung epithelial cells is serum-dependent .................. 47
3.2.4 LL-37 induces apoptosis in A549 epithelial cells ..................................................... 47
3.2.5 Effects of high-density lipoproteins and apolipoprotein A-1 on LL-37-induced cytotoxicity ......................................................................................................................... 49
3.2.6 Summary ..................................................................................................................... 53

4. DISCUSSION ..................................................................................................................... 56

REFERENCES ..................................................................................................................... 63
LIST OF FIGURES

Figure 1: Different structural classes of cationic peptides ............................................. 2
Figure 2: Structure and distribution of species-specific cathelicidins .................................. 4
Figure 3: Structural properties of LL-37 and LL-37B ....................................................... 24
Figure 4: Pattern of gene expression induced by LL-37 and LL-37B in A549 epithelial cells 26
Figure 5: LL-37 and LL-37B induce similar levels of IL-8 .................................................. 27
Figure 6: LL-37 and LL-37B induce similar release of lactate dehydrogenase (LDH) .......... 28
Figure 7: Localization of LL-37B in A549 epithelial cells .................................................. 30
Figure 8: Localization of LL-37 in A549 epithelial cells .................................................... 31
Figure 9: Uptake of LL-37B is an active process ............................................................... 32
Figure 10: Localization of LL-37B at higher concentration in A549 epithelial cells .......... 33
Figure 11: Brefeldin A causes characteristic disappearance of the golgi apparatus after 1h... 35
Figure 12: Uptake and trafficking of LL-37B in A549 epithelial cells .................................. 36
Figure 13: LL-37B does not co-localize with markers of endocytosis in A549 epithelial cells 37
Figure 14: A549 epithelial cells express formyl peptide receptor like-1 (FPRL-1) ............... 39
Figure 15: Uptake and localization of LL-37B does not involve Gi protein-coupled receptors 40
Figure 16: LL-37-induced IL-8 gene expression can be inhibited by brefeldin A .......... 41
Figure 17: LL-37B binds to two receptors on A549 epithelial cells ..................................... 43
Figure 18: The low affinity receptor for LL-37B appears to be formyl peptide receptor like-1 (FPRL-1) ................................................................. 44
Figure 19: Cytotoxic effects of LL-37 in different serum conditions as measured by lactate dehydrogenase (LDH) release ......................................................... 46
Figure 20: Localization of LL-37B differs in the presence of various types of serum .......... 48
Figure 21: LL-37 induces apoptosis in the presence of fetal bovine serum (FBS) but not human serum (HS) in A549 epithelial cells assessed by the TUNEL assay ............... 50
Figure 22: Effects of LL-37 on caspase-3 levels in A549 cells as measured by Western blot analysis ................................................................. 52
Figure 23: High density lipoprotein (HDL) but not apolipoprotein A-1 (Apo A-1) is able to protect against LL-37-induced cytotoxicity in the presence of 10% fetal bovine serum. 54

Figure 24: High density lipoprotein (HDL) but not apolipoprotein A-1 (Apo A-1) is able to protect against LL-37 induced cytotoxicity in the absence of serum. 55
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-1</td>
<td>apolipoproteinA-1</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6’-diamidino-2-phenylinodole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FPRL-1</td>
<td>formyl peptide receptor like-1</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoproteins</td>
</tr>
<tr>
<td>HS</td>
<td>human serum</td>
</tr>
<tr>
<td>IL-</td>
<td>interleukin-</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoproteins</td>
</tr>
<tr>
<td>LL-37B</td>
<td>biotinylated LL-37</td>
</tr>
<tr>
<td>LL-37C</td>
<td>LL-37 with an additional cysteine residue at the C-terminus</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl methyl sulfonyle fluoride</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>POPG</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>TBS with 0.1% Tween-20</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP digoxigenin nick end labeling</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENT

I would like to thank the many people that were there for me during these 3 long years. First of all, I would like to thank the Canadian Cystic Fibrosis Foundation for their monetary support. I would also like to thank Bob and the members of the Hancock lab. Donald, I could not have completed this without you – thanks for taking the time to train me at the beginning as well as providing valuable insights time and time again. Dawn, thanks so much for support and the advice you’ve given me as well as being a great friend outside of the lab. Monisha, you were always there to encourage me whenever things weren’t working out and thanks for helping with the microarray experiments. David, thanks again for helping me analyze the data for the binding kinetics. And last but not least, Danika, for introducing me to the cool world of microscopy.

Finally, I would like to thank my parents and my sister for their love and support. This thesis is dedicated to my dad, who was always there to encourage me on whatever endeavors I’ve chosen.
1. INTRODUCTION

1.1 Properties and function of cationic antimicrobial peptides

Cationic antimicrobial peptides are defined as peptides of 12 – 50 amino acids with a net positive charge of +2 to +7. These peptides have an excess of basic amino acids, such as lysine, arginine and histidine (Hancock, et al. 1999). There are four major structural classes that describe many of the known isolated peptides (Figure 1). The most common are unstructured peptides that fold into amphiphatic α-helical peptide upon contact with lipid bilayers and peptides that fold into β-sheets due to a conserved cysteine motif that form 3 disulfide linkages. Peptides that contain a high proportion of one or two amino acids (such as proline, tryptophan or histidine) usually have an extended structure. Looped peptides have a single intramolecular disulfide bond and depending on the location of the two cysteines, the size of the loop and tail can vary.

In 1966, Zeya and Spitznagel isolated the first cationic proteins from polymorphonuclear leukocytes (PMN) and noticed their antimicrobial activity against various pathogens (Zeya, et al. 1966). Since then, studies have shown the presence of cationic peptides in various species of animal, plant and insects. Cecropins A and B were first isolated from the silk moth Hyalophora cecropia. Cecropins were of particular interest in the pharmaceutical industry since they are toxic towards bacteria but not eukaryotic cells (Steiner, et al. 1981). However, the major component responsible for the immune response in the European honeybee Apis mellifera is melittin, which has the ability to kill prokaryotic as well as eukaryotic cells (DeGrado, et al. 1982).

In mammals, cationic peptides are the major components involved in the innate immune response and have also been implicated in regulating the adaptive immune response. These peptides are found in neutrophils and epithelial cells of various organs, including the lung, skin
Figure 1: Different structural classes of cationic peptides

Molecular models of cationic peptides from different structural classes. The charged regions of the peptides are shown in blue and the hydrophobic regions in green. (A) β-sheet structure, eg. human β-defensin-2; (B) amphipathic α-helical structure, eg. magainin 2; (C) β-turn loop structure, eg. bacternecin; (D) extended structure, eg. indolicidin. This figure was adapted from Hancock 2001.
and intestine and are thought to be a major component of host defence against infection on mucosal surfaces (Bals 2000, Hancock, et al. 2000).

Cationic peptides have been shown to be involved in many aspects of the innate immune response to infection. In addition to their broad-spectrum antimicrobial activity, these peptides have the ability to bind to bacterial inflammatory mediators (such as LPS and LTA) and inactivate the biological functions of these molecules as well as possessing anti-inflammatory properties (Bals, et al. 1999, Scott, et al. 2002). Peptides also have the ability to turn on specific macrophage responses and activate epithelial cells to secrete inflammatory mediators (such as MCP-1 and MCP-3) to recruit monocytes to the site of inflammation (Scott, et al. 2002).

1.2 Cathelicidins

Peptides of the cathelicidin family are synthesized as pre-pro-peptides and characterized by the conserved amino-terminal sequence of the peptide pro-piece and the variable carboxy-terminus (Zanetti, et al. 1995, Zanetti, et al. 2000). The pro-sequence is termed "cathelin" since this domain inhibits the activity of the first identified member of the cathelicidin family, cathepsin L (cathepsin L inhibitor). Molecules with a cathelin-like pro-peptide sequence have been isolated from multiple species including human, monkey, horse, cow, sheep, pig, rabbit and mouse (Zaiou, et al. 2002). The cathelin pro-sequence has been proposed to be involved in protecting the peptide from proteolysis during synthesis and trafficking of the peptide and/or mediating trafficking to the appropriate cellular compartment (Zaiou, et al. 2003). The human cathelicidin hCAP18 was first cloned from cDNA isolated from human bone marrow (Agerberth, et al. 1995). LL-37 is a proteolytically-processed form of hCAP18, that is released upon stimulation of cells and cleaved extracellularly by proteinase-3 (Sorensen, et al. 2001) (Figure 2). LL-37 is not only a major protein in the large granules of human neutrophils (Sorensen, et al. 1997) but is also produced by epithelial cells, including those in the squamous epithelium.
Figure 2: **Structure and distribution of species-specific cathelicidins**

The pre-processed form of the cathelicidins consists of a signal peptide at the N-terminus, a highly conserved cathelin domain and a variable cathelicidin domain. Cationic peptides with a cathelin-like pro-peptide sequence have been isolated from multiple species including humans. This figure was adapted from Zaiou et al 2002.
(Frohm Nilsson, et al. 1999), lung (Bals, et al. 1998), and by the epidermis, and is up-regulated in response to inflammatory stimuli (Frohm, et al. 1997). It can be found at unstimulated mucosal surfaces at concentrations of around 2 µg/ml, and at concentrations exceeding 1 mg/ml in inflamed epithelium (Bals, et al. 1998, Ong, et al. 2002). In addition, plasma has been reported to contain hCAP18 bound to lipoproteins at a concentration of 1.2 µg/ml (Sorensen, et al. 1999).

It has been demonstrated that LL-37 stimulates the expression of a wide variety of genes involved in the innate immune response, including those encoding chemokines (ie. IL-8 and MCP-1), differentiation factors, and anti-inflammatory cytokines (ie. IL-10) (Scott, et al. 2002). LL-37 has also been reported to be directly chemotactic for human neutrophils, monocytes and T-cells through formyl peptide receptor like-1 (FPRL-1, a G_i protein coupled receptor) (Yang, et al. 2000) and is also chemotactic for human mast cells using two different receptors, a high affinity (K_a = 2.3 µM) receptor that is not a G_i protein-coupled receptor and a low affinity, G_i protein-coupled receptor (K_a = 112 µM), but neither of these are the FPRL-1 receptor. It was also shown that LL-37 was found to induce IL-8 production through phosphorylation and activation of the mitogen activated protein kinases, ERK1/2 and p38 in human peripheral blood derived monocytes and human bronchial epithelial cell lines, but not in B- or T-lymphocytes, independent of the FPRL-1 receptor (Bowdish, et al. 2003, Tjabringa, et al. 2003). A proposed model suggests that LL-37 activates metalloproteinase, which cleaves membrane-anchored epidermal growth factor receptor (EGFR) ligands that activate EGFR resulting in ERK1/2 activation and gene transcription. This suggest that LL-37 may be one of the mediators that regulate the activity of epithelial cells in the lung by neutrophils (Tjabringa, et al. 2003). Recently, it has been shown that LL-37 has the ability to promote processing of the proinflammatory cytokine, IL-1β, via activation of P2X_7 receptors (Elssner, et al. 2004). Thus LL-37 is an important component of both the phagocyte and epithelial defence system in
humans, utilizing several different receptors. LL-37 also has a variety of other functions in immunity including promotion of mast cell histamine release (Niyonsaba 2001), stimulation of wound healing (Heilborn, et al. 2003), angiogenesis (Koczulla, et al. 2003) and modulation of dendritic cell differentiation (Davidson, et al. 2004).

LL-37 has been described as an antimicrobial peptide, but its activity against most bacteria is quite modest (Yan, et al. 2001, Zaiou, et al. 2003), and its antimicrobial activity may be confined to body compartments where the concentration is relatively high (e.g. neutrophils) or the ionic strength and divalent cation concentrations are reasonably low (e.g. gut). Nevertheless, despite its modest antibacterial activity (Gudmundsson, et al. 1996), exogenously added LL-37 protects mice against Staphylococcus aureus infections (M.G. Scott and R.E.W. Hancock, unpublished data), and transgenic overexpression of LL-37 in mouse airways resulted in decreased bacterial load and mortality following challenge with either Pseudomonas aeruginosa or Escherichia coli (Bals, et al. 1999). The ability of LL-37 to clear bacterial infections may reflect the effect of LL-37 on mechanisms of innate immunity. In addition the induction of anti-inflammatory gene products (Scott, et al. 2002) has an in vivo corollary in the ability of LL-37 to demonstrate potent protection against bacterial endotoxin (lipopolysaccharide) in animal models (Bals, et al. 1999, Scott, et al. 2002).

1.3 Receptor-mediated endocytosis and cellular transport

It has been shown repeatedly that some cationic peptides are internalized into eukaryotic cells by an unknown mechanism, although endocytosis is alluded to in several studies (Drin, et al. 2003, Richard, et al. 2003, Sandgren, et al. 2004). In this process, cells internalize macromolecules and particles as a means of communicating with their environment. This includes several diverse mechanisms such as phagocytosis and pinocytosis, which control entry of particles into the cells.
and have roles in clearing apoptotic cells during development, the immune response, neurotransmission, intercellular communications, signal transduction and cellular homeostasis.

With respect to immune activation, phagocytosis involves the uptake of large particles such as bacteria, yeast, apoptotic cells or cellular debris, leading to an activation of the cell’s inflammatory response. Phagocytosis is conducted by specialized cells, including macrophages, monocytes and neutrophils. On the other hand, pinocytosis in which cells take up nutrients, involves at least four basic mechanisms: macropinocytosis, caveolae-mediated endocytosis, clathrin- and caveolae-independent endocytosis and clathrin-mediated endocytosis (Conner, et al. 2003).

The most well understood component of pinocytosis is clathrin-mediated endocytosis, which is also known as receptor-mediated endocytosis. This process is crucial for intercellular communication during tissue and organ development and modulates signal transduction by controlling the levels of surface signaling receptors and clearance or downregulation of activated signaling receptors. High-affinity trans-membrane receptors and ligands concentrate at clathrin coated pits, on the plasma membrane, which invaginate and pinch off to form endocytic vesicles. The receptor-ligand complexes are carried into the cell and the clathrin coat on the vesicles is lost. The formation of the endocytic vesicles is not well understood and is thought to involve the GTPase mechanoenzyme, dynamin. The internalized proteins are then delivered to an early ‘sorting’ endosome, which requires Rab5 GTPase, early endosome antigen 1 (EEA1) and a lower pH (pH ~ 6.0). EEA1 is a Rab5 effector protein that is thought to be involved in endosome-endosome tethering and fusion. The lower pH releases the receptor from the ligand allowing the receptor to be recycled back to the cell surface by vesicles that bud from the endosome that target the plasma membrane. This permits the receptor to engage in additional binding/activity at the cell surface. Early endosomes containing the ligand convert to late endosomes while acquiring additional markers such as LAMP, mannose-6-phosphate receptor, Rab7-GTP and
lysobiosphophatidic acid, and undergo a further decrease in pH. The late endosome degrades the proteins further and then fuses with the lysosome, which is the endpoint of endocytosis. Lysosomes may deliver important material to late endosomes and then retrograde transport to the trans-golgi network/ER for eventual release into the cytosol (Conner, et al. 2003, Dautry-Varsat 1986, Jin, et al. 1993).

Microtubules have also been implicated in the movement of vesicles between organelles. Movement along microtubules, via kinesins and dynein, is relatively fast (~1 μm/s) and can occur over long distances (Apodaca 2001). Although receptor mediated endocytosis is not affected by microtubule depolymerization, there are examples where depolymerization agents affect cellular transport between early endosomes and late endosomes as well as movement between basolateral and apical surfaces of polarized epithelial cells (Jin, et al. 1993). In addition, actin filaments have been shown to be involved in endocytosis and vesicular transport (Schafer 2002, Stamnes 2002). Endocytosis is impaired on the apical surface of epithelial cells in the presence of actin-disrupting agents causing an increase in the number of clathrin-coated pits, however receptor-mediated endocytosis on the basolateral surface is unaffected. Therefore, this may indicate that actin is only required for internalization at the apical surface and that actin is not an absolute requirement for receptor-mediated endocytosis. It is not entirely clear if and how actin is involved in receptor-mediated endocytosis, as it has been recently shown, using an actin-stabilizing agent, that endocytosis from the basolateral surface of MDCK and T84 cells also involves actin. The reason for the differences seen between the apical and basolateral surfaces may reflect the differences in the actin pools at each of these surfaces of polarized epithelial cells (Apodaca 2001). Transport of endocytic vesicles within the cell is powered by actin polymerization forming a comet-like tail, reminiscent of those assembled by Listeria (Zhang, et al. 2002).
1.4 Cytotoxicity and cell death

There have been reports regarding the cytotoxicity of some cationic peptides. For instance, LL-37 was found to be cytotoxic to both prokaryotic and eukaryotic cells (Johansson, et al. 1998, Oren, et al. 1999). The two bovine cathelicidins, BMAP-27 and BMAP-28, were also demonstrated to be cytotoxic for both cells lines and fresh hematopoietic tumor cells, but not on resting lymphocytes. This observed cytotoxicity is associated with cationic peptide-induced membrane permeabilization, as assessed by propidium iodide uptake, and is followed by programmed cell death (Risso, et al. 1998, Skerlavaj, et al. 1996).

Programmed cell death, also known as apoptosis, is required for normal development and maintenance of tissue physiology in addition to having a role as a defense mechanism to remove damaged cells. There are many prominent morphological features that accompany apoptosis, such as membrane blebbing, cell shrinkage and nuclear fragmentation. These events take place over a prolonged period of time while the cell retains the integrity of its plasma membrane and organelles, thus protecting against proinflammatory responses in the surrounding tissues (Kerr, et al. 1972). It is important to note that apoptosis is an immunologically silent process where immunosuppressive cytokines, such as IL-10 and TGF-β are produced, and phagocytes rapidly engulf the apoptotic bodies to prevent further immunological responses (Maderna, et al. 2003). However apoptotic cells may become necrotic when the apoptotic cells cannot be cleared in diseases such as cystic fibrosis (Hudson 2001).

There are two main pathways by which apoptosis can be activated, the extrinsic pathway and the intrinsic pathway, both of which involve cysteine aspartate-specific proteases, termed caspases. The activation of the extrinsic pathway involves the binding of death receptors to their ligand. The death receptor is characterized by a C-terminal intracellular death domain (DD), which serves to recruit adaptor proteins, which also contain a DD. The initiator pro-caspases (pro-caspase 2 or 8) are then recruited to the activated receptor via the death effector domain.
(DED) or the caspase activation and recruitment domain (CARD), which bind to the DD, forming the death-inducing signaling complex (DISC). The formation of this complex then leads to downstream activation of executioner pro-caspases (procaspase 3, 6 and 7) (Denecker, et al. 2001). Interestingly, it has been recently shown that the apoptotic signaling pathway can be activated through other receptor-mediated mechanisms including the activation of caspase-1 through Toll-like receptor 2, which has bacterial lipoproteins as its ligand. The adaptor molecule, myeloid differentiation factor 88 (MyD88) binds Fas-associated death domain protein (FADD) resulting in the activation of pro-caspase-8 (Aliprantis, et al. 2000).

The second pathway is the intrinsic or mitochondrial pathway, which is triggered by developmental programs and environmental stress including chemotherapeutic agents, UV radiation and stress molecules such as reactive oxygen and nitrogen species. The mitochondrial pathway is activated when there is a decrease in the mitochondrial transmembrane potential due to the disruption of membrane integrity, which is controlled by the Bcl-2 family of proteins. As a consequence of this disruption, cytochrome C is released from the mitochondrial intermembrane space to the cytosol. Cytochrome C binds dATP/ATP and Apaf-1, which form the apoptosome complex. Once formed, Apaf-1 undergoes a conformational change revealing its CARD domain, which activates pro-caspase-9, leading to the downstream activation of pro-caspase-3 and executioner pro-caspases in the cascade (Regula, et al. 2003).

To facilitate engulfment by phagocytes, apoptotic cells pump out ions (K⁺, Cl⁻ and organic osmolytes) and reorganize their cytoskeleton, which is usually accompanied by nuclear condensation, leading to a decrease in cell volume. Nuclear condensation consists of chromatin condensation (pyknosis) and nuclear fragmentation (karyohexis). Caspase-3 and an unknown protease activate acinus inducing chromatin condensation without causing DNA fragmentation. Apoptosis-inducing factor (AIF), which normally resides in the mitochondrial intermembrane, is translocated to the nucleus during apoptosis to cleave DNA to generate high molecular weight
DNA fragments, which are further degraded into approximately 180-bp fragments by caspase-activated DNase (CAD) (Denecker, et al. 2001, Maderna, et al. 2003).

Necrosis is a form of cell death, distinct from apoptosis. Necrosis has distinct morphological features including cytoplasmic swelling, organelle breakdown and rapid plasma membrane rupture leading to an inflammatory response (Kerr, et al. 1972). Historically, necrotic cell death was considered to be an accidental type of death caused by gross cell injury resulting in death of groups of cells within tissues. However, it has been recently shown that signaling pathways, such as death receptors, the caspase cascade, and the mitochondrial pathway participate in both necrosis and apoptosis and, as a result, allow cells to switch between the two pathways. The decision points between the two pathways include short or prolonged opening of the mitochondrial pore, the extent of oxidative stress, the concentration of cellular ATP content and whether there are high or low levels of Bcl-2 present (Denecker, et al. 2001, Eguchi, et al. 1997, Tsujimoto, et al. 1997). Therefore, necrosis, along with apoptosis, may be considered as a form of programmed cell death and the disturbance of the balance between these two processes may lead to the development of disease.

1.5 Aims of this study

The aim of this thesis was to characterize the interaction between the human cathelicidin, LL-37, and cells involved in innate immunity and to examine the resulting consequences. Since other cationic peptides have been shown to interact directly with effector cells involved in innate immunity, I hypothesize that LL-37 interacts with human epithelial cells in a receptor-mediated fashion and that the activities of LL-37 can be regulated by human serum components. Although the effects of LL-37 on eukaryotic cells have been studied, the mechanism of how LL-37 interacts with eukaryotic cells is not well understood.
2. MATERIALS AND METHODS

2.1 Peptide synthesis

LL-37 (LLGDFRKSKEKIGKEFKRIVQRKDFLRNLVPRTES) and LL-37C (LLGDFRKSKEKIGKEFKRIVQRKDFLRNLVPRTESC) were synthesized at the Nucleic Acid/Protein Synthesis (NAPS) Unit at UBC by Fmoc [(N-(9-fluorenyl) methoxycarbonyl)] chemistry using an Applied Biosystems Model 431 peptide synthesizer. Peptide purity was determined by using amino acid analysis.

2.2 Biotinylation of LL-37

LL-37C was biotinylated at the C-terminal cysteine side chain with N-\(\alpha\)-(3-maleimidylpropionyl) biocytin (Molecular Probes, Eugene, OR). LL-37C (2 mmol) and N-\(\alpha\)-(3-maleimidylpropionyl) biocytin (23 mmol) were dissolved separately (430 mM and 1 mM respectively) in 50 mM Tris buffer, pH 7 and mixed. The mixture was incubated for 2 h at room temperature with shaking. Excess N-\(\alpha\)-(3-maleimidylpropionyl) biocytin was quenched using 2-mercaptoethanol (BioRad, Montreal, QC). Biotinylated LL-37 (LL-37B) was purified using a reversed phase FPLC column (Resource 15RPC 3ml, Pharmacia, Piscataway, NJ) with a water/acetonitrile gradient containing 0.1% trifluoroacetic acid. The yield of LL37B after purification was 66% as determined by amino acid analysis. Peptide purity was confirmed by HPLC and MALDI-TOF mass spectroscopy.

2.3 Cell line maintenance

The human epithelial cell line A549 was obtained from American Type Culture Collection (ATCC, Manassas, VA) (Giard, et al. 1973). The cell line was maintained in complete DMEM (Dulbecco’s Modified Eagle Medium, Invitrogen, Burlington, ON), which was
DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT), and incubated at 37°C and 5% CO₂ in cell culture flasks (Costar, Cambridge, MA). The cells were passaged at least twice a week by treating the monolayer with Trypsin-EDTA (Invitrogen) at 37°C for 5 min to dissociate the cells from the flask. The detached cells were transferred to a 50 ml centrifuge tube containing 20 ml complete DMEM medium and then centrifuged for 5 min at 135 x g. The supernatant was discarded and the cells were resuspended in DMEM complete medium. A 175 cm² flask was seeded with 10⁶ viable cells in 40 ml DMEM complete medium and incubated at 37°C in 5% CO₂ for 7 days.

2.4 Liposome preparation

A chloroform solution of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) (Avanti Polar Lipids Inc., Alabaster, AL), or an equimolar mixture of both lipids, was dried under a stream of N₂. Residual solvent was removed by desiccation under vacuum for 2 h. The resulting lipid film was re-hydrated in 10 mM phosphate buffer (pH 7.0). The suspension was put through five cycles of freeze-thaw to produce multilamellar liposomes, followed by extrusion through 0.1 μm double-stacked Poretics filters (AMD Manufacturing Inc., Mississauga, ON) using an extruder device (Lipex Biomembranes, Vancouver, BC).

2.5 Circular dichroism spectrometry

Circular Dichroism (CD) spectra were obtained using a Jasco J-810 spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan). Each spectrum (190-250 nm) was the average of 10 scans using a quartz cell of 1 mm path length at room temperature. The scanning speed was 50 nm/min with a step size of 0.1 nm, a response time of 2 s, and a 1 nm bandwidth. The peptide concentrations utilized were 4.0 μM for LL-37 and 4.3 μM for LL-37B in 10 mM Tris buffer.
(pH 7.4). The concentration of lipid was 1 mM (POPC:POPG at a molar ratio of 1:1). Spectra were baseline corrected by subtracting a blank spectrum of a sample containing all components except the peptide. Ellipticities were converted to mean residue molar ellipticities \([\theta]\) in units of deg\(\times\)cm\(^2\)/dmol.

2.6 LDH assay for cytotoxicity

A549 cells were maintained and passaged as described above. The epithelial cells were seeded in 24 well plates at a density of \(10^{5}\) cells/well in Complete DMEM medium and incubated at 37\(^\circ\)C in 5\% CO\(_2\) overnight. The medium was removed from the cells and replaced with fresh complete medium. Cells were treated with LL-37 or LL-37B at a range of concentrations (5 – 300 \(\mu\)g/ml) for 24 h. Lactate dehydrogenase is a cytoplasmic enzyme that is present in all cells and is released into the cell culture supernatant when the plasma membrane is damaged. Two wells of cells were treated with 1\% Triton X-100, resulting on complete cell lysis, as a positive control for maximum LDH release, while media alone was set to 0\% LDH release. After 24 hour incubation at 37\(^\circ\)C in 5\% CO\(_2\), the supernatants were removed, centrifuged at 4500 \(\times\) g for 5 min to remove contaminating cells and the level of LDH was assayed in duplicate using a colorimetric Cytotoxicity Detection kit (Roche, Mannheim, Germany). The absorbance was read at 490 nm and 600 nm (as reference wavelength) using a Biotek reader.

2.7 Cytokine production in A549 epithelial cells

The human epithelial cell line A549 was obtained from the American Type Culture Collection (Manassas, VA). The A549 epithelial cell line was maintained in complete DMEM. A549 cells were seeded in 24 well plates at a density of \(10^{5}\) cells per well in complete DMEM and incubated overnight at 37\(^\circ\)C, 5\% CO\(_2\). Media was removed from cells grown overnight and replaced with fresh complete DMEM. Cationic peptides were added and cells further incubated
for 24 h at 37°C, 5% CO₂. Supernatant was removed and quantified for IL-8 by ELISA as per the manufacturer’s directions (Biosource, Montreal, QC). Absorbance was read at 450nm using a Biotek plate reader.

2.8 RNA isolation

A549 cells were placed in 150 mm tissue culture dishes at 10⁶ cells/dish in complete DMEM, and incubated overnight at 37°C in 5% CO₂. DMEM was removed from cells grown overnight and fresh medium was added and then incubated with peptides for 4 h. After stimulation, cells were washed with PBS. Total RNA was isolated as per the manufacturer’s directions using RNAqueous (Ambion, Austin, TX). RNase inhibitor (Ambion) was added to the RNA sample to prevent RNA degradation. To remove contaminating genomic DNA, the RNA sample was incubated with DNase I (Ambion) for 30 min at 37°C. DNase I was subsequently inactivated using DNase inactivation reagent (Ambion). RNA quality and quantity was assessed using a Bioanalyzer (Agilent, Palo Alto, CA) and visualization by electrophoresis on a 2% agarose gel.

2.9 Microarrays

14K human oligonucleotide expression arrays, which were printed on glass slides using the 70-mer library PRHU04 from Qiagen (Venlo, Netherlands; see www.operon.com for details of the library). The microarray slides were obtained from the Genome BC Array Facility (Vancouver Hospital, Vancouver, BC). cDNA probes were prepared from 2 µg of total cellular RNA by reverse transcription and labeled with either biotin or fluorescein by using a Qiagen LabelStar array kit labeling module as per the manufacturer’s instructions. The cDNA probes were then purified using the cleanup module of the same kit. Microarray slides were subsequently hybridized using the RLS array detection system (Invitrogen/Genicon, San Diego,
CA), as per the manufacturer’s instructions. The array hybridization image was captured using GSD-501 RLS detection and imaging instrument (Invitrogen/Genicon). The hybridization signals were then quantified using ArrayVision Version 8.0 (Imaging Research Inc., St. Catherines, ON) and analyzed by GeneSpring (Silicon Genetics, Redwood City, CA). The data was normalized using the 50th percentile parameter of GeneSpring. All results were derived from 3 independent experiments, each involving two technical replicates obtained through dye swapping. All experimental information and procedures were entered into a MIAME compliant database.

2.10 Immunofluorescence

A549 cells were seeded onto 12 mm diameter coverslips (VWR, Edmonton, AB) at a density of $10^5$ cells per coverslip in complete DMEM (see above) and incubated overnight at 37°C, 5% CO2. The following day, media was removed from cells grown overnight and replaced with fresh complete DMEM, DMEM containing 10% human serum (Sigma) or DMEM without serum supplementation. For experiments at low temperature, cells were maintained for 15 min on ice before adding peptide and kept on ice throughout the experiment. Biocytin was used as a negative control for all conditions. LL-37 or LL-37B were incubated with cells from 30 min to 4 h at 4°C and 37°C, 5% CO2. Following peptide incubation, the coverslips were washed with phosphate-buffered saline (PBS) and fixed with 4% para-formaldehyde (PFA). The coverslips were washed extensively after fixing and the cells were permeabilized using 0.1% Triton X-100 in PBS, and LL-37 was revealed with α-LL-37 clone 3D11 (1/200 dilution of 1 mg/ml) and visualized using an Alexa-conjugated secondary antibody (1/200 dilution of 2 mg/ml, Molecular probes, Eugene, OR). LL-37B was probed using streptavidin (1/10 dilution of 1 mg/ml, Molecular Probes) and then detected with biotin-Oregon green (1/10 dilution of 1 mg/ml, Molecular Probes). Actin was detected using Alexa-conjugated phalloidin (300U, Molecular probes). Cells were then washed extensively with PBS and the coverslips were mounted in
Vectashield with 4',6'-diamidino-2-phenylindole (DAPI) (1.5 μg/ml, Vector Laboratories, Burlingham, CA) to stain for host cell DNA. Coverslips were viewed using a BioRad radiance confocal microscope.

2.11 Inhibition studies with immunofluorescence

In order to determine which components were required for the trafficking of LL-37B, A549 cells were prepared as described above for immunofluorescence and various inhibitors were used. Cells were treated with nacodazole, which induces microtubule polymerization, at 2 μg/ml for 30 min prior to the addition of LL-37B. Actin polymerization was blocked using cytochalasin D at 2 μg/ml (Sigma, St Louis, MO) for 30 min prior to the addition of LL-37B. To inhibit G-protein coupled receptors, cells were treated with pertussis toxin (Ptx, List Biological Laboratories Inc., Campbell, CA) at 100 ng/ml for 2 h prior to LL-37B addition. The FPRL-1 agonist, WKYMVM (W-peptide; a generous gift from Dr. Claes Dahlgren, Phagocyte Research Laboratories, Dept. of Rheumatology and Inflammation Research, University of Goteborg, Sweden) was added at 50 nM or 500 nM together with LL-37B, to compete for FPRL-1 receptor binding. Endocytosis was inhibited by treating A549 cells with brefeldin A (5 μg/ml) (Sigma) for 1 hr prior to the addition of LL-37B. Biocytin (Sigma) was used as a negative control. Cells were incubated with LL-37B for 30 min to 4 h at 4°C or 37°C, 5% CO₂. The cells were fixed and probed with streptavidin (Molecular Probes) and Alexa-conjugated phalloidin (Molecular Probes). Coverslips were mounted as described above for immunofluorescence. Coverslips were viewed using a BioRad radiance confocal microscope (Hercules, CA).
2.12 **Co-localization studies with endocytic markers**

In order to detect which components of the endocytic pathway were involved, A549 cells were prepared as described above for immunofluorescence and various endocytic markers were used. Following fixation and extensive washing with PBS, cells were permeabilized with 0.2% saponin (Sigma) in PBS and then probed with antibodies specific for components of the endocytic transport mechanism along with streptavidin (Molecular probes). The coverslips were washed extensively with 0.2% saponin in PBS and probed with Alexa-conjugated antibodies and biotin-Oregon green (Molecular probes). The coverslips were mounted in VectaShield with 4',6'-diamidino-2-phenylindole (DAPI) to stain for host cell DNA (Vector Laboratories). Coverslips were viewed using a BioRad Radiance confocal microscope.

---

2.13 **Binding assays**

A549 cells were placed in 96-well plates in complete DMEM, at a concentration of $1 \times 10^4$ cells/well and incubated overnight at $37^\circ$C in 5% CO$_2$. Media was then removed from the wells and in order to block uptake, the cells were treated with brefeldin A (5 μg/ml in complete DMEM) for 1 hr prior to the addition of LL-37B. For competition studies the FPRL-1 agonist peptide, WKYMVM (W-peptide, a generous gift from Dr. Claes Dahlgren, Phagocyte Research Laboratories, Dept. of Rheumatology and Inflammation Research, U. Goteborg, Sweden) was added at the same time as LL-37B. Cells were incubated with LL-37B (and W-peptide for the competition studies) for 15 min at $37^\circ$C. Following incubation, the cells were gently washed with PBS and incubated with streptavidin-HRP (1/200 dilution, R&D system, Minneapolis, MN) at $23^\circ$C for 60 min. Cells were washed extensively with PBS and then incubated with 3,3',5,5'-Tetramethylbenzidine Liquid Substrate (TMB, Sigma, St Louis, MO) at room temperature for 30 min and protected from light. The plates were read with a Bio-Tek plate reader at an absorbance wavelength of 450 nm (correcting background by subtracting the absorbance at 570 nm). The
B\textsubscript{max}, K\textsubscript{d} and approximate receptor numbers were obtained by Scatchard analysis using GraphPad (GraphPad Software Inc., San Diego, CA)

### 2.14 Antibodies

Murine monoclonal α-golgin 97, used at 1/200 dilution, was obtained from Molecular Probes; rabbit monoclonal α-calnexin, used at 1/1000 dilution, was obtained from Sigma; murine monoclonal α-LAMP-2, used at 1/200 dilution was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA); murine monoclonal α-nucleolin, used at 1/200 dilution, was obtained from Molecular Probes; anti-LL-37 clone 3D11, used at 1/200 dilution, was a generous gift from Dr. Pieter S. Hiemstra and G. Sandra Tjabringa, Department of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands; α-cathepsin D, used at 1/200 dilution, was obtained from Scripps Laboratories (San Diego, CA); Alexa-conjugated secondary antibodies, used at 1/200 dilution, were obtained from Molecular Probes; α-caspase-3, used at 1/200 dilution, was obtained from Stressgen Bioreagents (Victoria, BC); α-actin used at 1/10000 dilution, was obtained from Sigma; α-rabbit-HRP and α-mouse-HRP, used at 1/500 dilution, was obtained from Cell Signalling Technologies (Beverly, MA)

### 2.15 Real-time quantitative RT-PCR

RNA was isolated as described above. One μg of total RNA was reverse transcribed into cDNA using SuperScript II\textsuperscript{Tm} (Invitrogen, Carlsbad, CA). A ten-fold dilution of the resulting cDNA was made and 5 μl was used for each real-time PCR reaction. Primer/probes for IL-8 (Ref Seq NM 000584) and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Ref Seq NM 002046), were purchased from the manufacturer (Assay on Demand, Applied Biosystems Inc, Foster City, CA). PCR reactions were performed in an ABI Prism 7000
sequence detector (TaqMan, Applied Biosystems, Foster City, CA). Each PCR amplification was performed in duplicate using the following conditions: 15 s at 95°C and 1 min at 60°C for 45 cycles.

Semi-quantitative RT-PCR was also performed to determine if A549 epithelial cells express FPRL-1. RNA was isolated as mentioned above and 1 µg of total RNA was reverse transcribed into cDNA using SuperScript II™ (Invitrogen). The resultant cDNA was used as a template in PCR for FPRL-1 gene (5'-CCTAATGCCAGTTCCAGCTTC-3', 5' - ATTTCCTTAAGCTTTACC-3'). RT-PCR reaction was performed in duplicate using the following conditions: one cycle of 15 s at 94°C, 28 cycles of 94°C for 15 min, 57°C for 30 s and 72°C for 40 s and finally one cycle of 72°C for 10 min.

2.16 TUNEL assay

A549 cells were seeded onto 12 mm diameter coverslips at a density of $10^5$ cells per coverslip in complete DMEM (see above) and incubated overnight at 37°C, 5% CO₂. The following day, media was removed from the cells grown overnight and cells were washed with PBS before the addition of fresh complete DMEM or DMEM with 10% HS. Ten – 100 µg/ml LL-37 was added to the cells and incubated for 24 h. TUNEL-assay was performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche). Briefly, after peptide incubation, cells were washed with PBS and fixed with 50% acetone/50% methanol for 20 min. Coverslips were washed extensively with PBS, permeabilized with 0.1% Triton X-100 and then incubated with TUNEL reaction mixture at 37°C for 60 min, as per the manufacturer’s instructions. Coverslips were washed with PBS and mounted with Vectashield containing DAPI (Vector Laboratories). Coverslips were viewed using a Zeiss fluorescence microscope. Quantitation was done by counting cells in 3 fields of view.
2.17 Western blot analysis

A549 cells were seeded in a 6-well plate at $2 \times 10^5$ cells/2ml and grown overnight in complete DMEM. Complete DMEM was removed from the cells grown overnight and replaced with fresh medium. They were then incubated with concentrations of LL-37 ranging from $10 - 100 \mu g/ml$ for 24 hrs. After stimulation, supernatant was removed and cells were washed with sterile PBS. After washing, the cells were solubilized with pre-heated (95°C) 5X sample buffer (300mM Tris-HCl pH 6.8, 0.5M DTT, 12% SDS, 10% glycerol). Cell lysates were then incubated at 95°C for 10 minutes before loading onto 14% SDS-PAGE and then transferred to nitrocellulose membranes. The membrane was blocked for 1 hr with 5% skim milk powder and then incubated overnight at 4°C with primary antibody in TBS + 0.05% Tween 20 (TBST). Membranes were rinsed with TBST and then incubated for 2 hrs at room temperature with secondary antibody conjugated to horseradish peroxidase. After washing the membranes for 30 min with TBST, immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection (Amersham, Piscataway, NJ). Membranes were re-probed with actin, as a loading control. Quantitation of bands was done using ImageJ (NIH, Bethesda, MD).

2.18 HDL/Apolipoprotein A-1 isolation from whole blood

Blood was taken from healthy individuals into sodium heparin Vacutainer collection tubes (BD Biosciences, Mississauga, Ontario) according to the University of British Columbia clinical research ethics board protocol. Blood samples were centrifuged for 25 min at 1740 rpm. Plasma was transferred into 50 ml conical tubes. Each donor was handled separately. Briefly, plasma volumes were adjusted to 25 ml with PBS and the following was added: 0.04% ethylene diamine tetraacetic acid (EDTA, Sigma), 0.005% gentamicin sulphate (Sigma), 0.05% sodium azide (NaN₃, Sigma) and 0.015% phenylmethanesulfonyl fluoride (PMSF, Sigma). The resulting solution was adjusted to a density of 1.063 g/ml with potassium bromide (KBr, Sigma) and then...
centrifuged for 24 h at 165 000 x g, 4°C. The low density lipoprotein (LDL) layer was removed. The volume of the resulting solution was re-adjusted to 25 ml with PBS and density to 1.21 g/ml with KBr (Sigma) and centrifuged for 24 h at 165 000 x g, 4°C. The high density lipoproteins (HDL) layer was removed and the HDLs were delipidated with a 2:1 chloroform:methanol solution to obtain apolipoprotein A-1 (Edelstein, et al. 1986, Schumaker, et al. 1986). Each fraction was run on an 8% SDS-PAGE gel to confirm lipoprotein/protein isolation.

2.19 Statistical analysis

Results are generally expressed as the mean ± standard error (SEM) of 3 independent experiments. The Student’s two tailed t-test was used to test significance.
3. RESULTS

3.1 Interaction and cellular localization of the host defence peptide, LL-37, in lung epithelial cells

3.1.1 Introduction

Although the effects of LL-37 on eukaryotic cells had been studied, the mechanism of how LL-37 interacts with eukaryotic cells was not well understood. In this chapter, the interaction between LL-37 and lung epithelial cells was characterized. Using biotinylated LL-37 and confocal microscopy, in conjunction with specific inhibitors, the mechanism of uptake and localization of LL-37B was demonstrated. Binding assays were also performed to determine whether A549 epithelial cells have specific receptor(s) for LL-37B.

3.1.2 Biotinylated LL-37 and LL-37 have similar structural and biological properties

Circular dichroism (CD) spectroscopy was used to compare the secondary structures of LL-37 and LL-37B in aqueous solution and in the presence of POPC:POPG (1:1) large unilamellar vesicles. The CD spectra are shown in Figure 3. Both LL-37 and LL-37B were largely unstructured in free solution as indicated by the minima near 205 nm. Upon the addition of lipid vesicles both peptides adopted an α-helical structure, as indicated by the two minima at 210 nm and 222 nm and the maxima at 195 nm. The similarity of the secondary structure of LL-37 and LL-37B in both the absence and presence of a membrane environment indicates that the structure and membrane-binding properties of LL-37 were not affected by biotinylation. LL-37 and its biotinylated counterpart were also tested in various biological assays to confirm their functional similarity. A549 cells were exposed to either 10 μg/ml LL-37 or LL-37B for 4 h and the RNA was isolated and converted to labeled cDNA and used to probe human oligonucleotide
Figure 3: Structural properties of LL-37 and LL-37B

CD spectra of 4 μM LL-37 and 4.3 μM LL-37B in 10 mM Tris-HCl buffer, pH 7.4 in the presence and absence of 1 mM POPC:POPG (1:1) liposomes.
microarrays for patterns of gene expression. Of the 14,000 genes for which expression levels were tested, only 14 genes had expression levels that differed by greater than 4-fold between the two peptides (Fig. 4). This was a control experiment demonstrating that the patterns of gene expression were very similar for both peptides. LL-37 has been previously demonstrated to induce the production of IL-8, a potent neutrophil chemokine, in lung epithelial cells (Scott, et al. 2002, Yang, et al. 2001). IL-8 production in A549 cells was studied by ELISA in response to treatment by LL-37 or LL-37B at a range of concentrations. After 24 h of stimulation, there was a dose dependent increase in IL-8 induction between 10 and 50 µg/ml LL-37/LL-37B, with no significant difference observed between the two peptides (p > 0.05, Fig 5).

It has been well documented that LL-37 can be cytotoxic to certain eukaryotic cell lines at high concentration (Oren, et al. 1999), although recently published data demonstrates that LL-37 is not cytotoxic to human primary monocytes at the concentrations used in this study (Bowdish, et al. 2003). In order to determine the level of cytotoxicity exhibited by LL-37, lactate dehydrogenase assay (LDH) release experiments were performed. There were no significant differences (p > 0.05) in LDH release between LL-37 to LL-37B. At the concentration (10 µg/ml) used for most of these studies, no significant LDH release was observed. At the highest concentrations studied here (50 µg/ml), I observed 35-44% LDH release from the A549 epithelial cells (Fig 6).

3.1.3 Uptake of LL-37B into cells is an active process resulting in localization to the perinuclear region

The human lung epithelial cell line A549 was incubated with 10 µg/ml of LL-37B at 37°C, 5% CO₂ for various lengths of time. LL-37B was internalized by the cells and at 30 min was detected
Figure 4: Pattern of gene expression induced by LL-37 and LL-37B in A549 epithelial cells

A549 cells were incubated with LL-37 or LL-37B for 4 hours, the RNA was harvested, and total RNA was used to make labeled cDNA probes (biotin or fluorescein) and hybridized onto microarrays spotted with 14,000 human genes in duplicate and labeled as described in Materials and Methods. The RLS images were quantified, the densities were normalized and the average intensities for LL-37 were plotted against those of LL-37B on a log scale. The plot represents averages of 3 biological replicates and 2 technical replicates for each biological replicate.
Figure 5: LL-37 and LL-37B induce similar levels of IL-8

A549 cells were stimulated with increasing concentration of LL-37 (□) and LL-37B (■) for 24 h. The supernatants were tested for IL-8 by ELISA. Results are expressed as mean ± standard error of 3 separate experiments. Student’s two-tailed t-test was performed. *, p < 0.05.
Figure 6: LL-37 and LL-37B induce similar release of lactate dehydrogenase (LDH)

A549 cells were stimulated with increasing concentration of LL-37 (□) and LL-37B (■) for 24 h in the presence of 10% fetal bovine serum in DMEM. The supernatants were tested % lactate dehydrogenase as described in Methods and Materials. 100% LDH release was the amount of LDH released into the supernatant when 1% Triton X-100 was incubated with cells. 0% LDH release was taken as the amount of LDH in media alone. Negative control was subtracted as background. Results are expressed as mean ± standard error of 3 separate experiments. Student’s two-tailed t-test was performed. *, p < 0.05.
in small circular structures reminiscent of vesicles throughout the cytosol. By 1 h, most of these vesicles had congregated around the perinuclear region. At 4 h, the majority of the peptide was localized around the perinuclear region with some peptide accumulating around the nuclear membrane (Fig. 7). Similar observations were made using unlabelled LL-37, visualized with specific antibody (Fig. 8), although in this case since labeling was direct, the signal strength was somewhat lower. To determine whether LL-37 was taken up into cells through an active or passive mechanism, A549 cells were incubated with 10 μg/ml LL-37B at 4°C. Under these conditions LL-37B bound to and co-localized with actin at the edge of the cell after 4 h (Fig. 9), indicating that uptake into cells was temperature dependent, as expected if uptake was dependent on a receptor-mediated mechanism.

At a higher, somewhat cytotoxic concentrations of LL-37B (50 μg/ml), the peptide was localized to the nucleus and around the nuclear membrane of cells after 4 h (Fig. 10).

3.1.4 Uptake of LL-37B incorporates elements of endocytosis, and LL-37 trafficking is mediated by microtubules

Three separate experiments were performed to determine whether LL-37B was entering cells via an endocytotic pathway. The effects of specific inhibitors on intracellular accumulation and localization were investigated. Brefeldin A is a fungal metabolite that is widely utilized as an endocytosis inhibitor but has been reported to have multiple effects, dependent on cell type and species (Pellham 1991, Sciaky, et al. 1997). This compound targets ADP-ribosylation factor GTPase exchange factor in the secretory pathway, inhibiting protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus. It also affects endocytosis and related events such as vesicular assembly and secretion and antigen presentation. Consistent with this, confocal microscopy demonstrated that after 1h of treatment, brefeldin A caused the characteristic disappearance of the Golgi apparatus in A549 cells (Figure 11). To determine if the
Figure 7: Localization of LL-37B in A549 epithelial cells

A549 cells were incubated with 10 μg/ml (2.2 μM) LL-37B for 30 min, 1 h, 2 h and 4h at 37°C. Cells were prepared for immunofluorescence as described in Methods and Materials. Right panels represent a merge of LL-37B (left panels, green) and actin (middle panels, red). Bars represent 20 μm.
Figure 8: Localization of LL-37 in A549 epithelial cells

Localization of LL-37B was confirmed by incubating A549 epithelial cells with 10 µg/ml (2.2 µM) LL-37 for 30 min, 2 h and 4 h at 37°C. Cells were prepared for immunofluorescence as described in Methods and Materials. Right panels represent a merge of LL-37 (left panels, green) and actin (middle panels, red). Bars represent 20 µm.
Figure 9: Uptake of LL-37B is an active process

A549 epithelial cells were pre-incubated on ice 15 min prior to the addition of 10 µg/ml (2.2 µM) LL-37B for 4 h at 4°C. Cells were prepared for immunofluorescence as described in Materials and Methods. Right panel represent a merge of LL-37B (left panel, green) and actin (middle panel, red). Bars, 20 µm.
A549 epithelial cells were incubated with 50 µg/ml (11 µM) LL-37B for 4 h at 37°C. Cells were prepared for immunofluorescence as described in Methods and Materials. Right panel represents a merge of LL-37 (left panel, green) and actin (middle panel, red). Bars represent 20 µm.
internalization of LL-37B was through an endocytic mechanism, cells were pretreated with brefeldin A for 1 h prior to the addition of 10 μg/ml LL-37B. In brefeldin A-treated cells after 4 h, there was no intracellular accumulation of LL-37B, and the majority of the peptide was seen on the outside of the cell (Figure 12A), consistent with the requirement for functional endocytic pathways for the uptake of LL-37.

To determine if endocytosis of LL-37B was dependent on actin polymerization, A549 cells were pre-treated for 30 min with cytochalasin D, which binds G-actin and prevents the polymerization of actin monomers, prior to the addition of 10 μg/ml LL-37B. There was no observable difference in the uptake and accumulation of LL-37B after 4 h (Fig. 12B) demonstrating that uptake and trafficking of LL-37 did not require the polymerization of actin. However, when cells were treated with nocodazole, which depolymerizes microtubules, the accumulation of peptides to the perinuclear region did not occur (Fig. 12C). Under these conditions, after 4 h, LL-37B was retained close to the periphery of the cell, rather than migrating to a perinuclear location as observed with control cells. These results are consistent with the conclusion that the trafficking of LL-37B from the cell periphery to the perinuclear region is mediated by microtubules. Co-localization studies were also performed with endocytic markers that included LAMP2, golgin-97, nucleolin, calnexin and cathepsin D, at 15, 30 min, 1, 2 and 4 h, but no co-localization was observed (Figure 13).

3.1.5 Uptake of LL-37B is not mediated by G protein-coupled receptors

LL-37 was recently proposed be chemotactic for macrophages, neutrophils and T-lymphocytes via a G protein-coupled receptor, FPRL-1, and mast cells, by a G protein-coupled receptor that was not FPRL-1 (Niyonsaba, et al. 2002, Yang, et al. 2000). Other events including induction of the chemokine IL-8 have been shown to be independent of the G protein-coupled
Figure 11: Brefeldin A causes characteristic disappearance of the golgi apparatus after 1h

A549 cells were incubated with (A) and without (B) 5 mg/ml brefeldin A for 1h. Cells were prepared for immunofluorescence as described in Materials and Methods. The right panels represent a merger of golgi (left panel, red), actin (middle panels, green). Bars represent 20 μm.
Figure 12: Uptake and trafficking of LL-37B in A549 epithelial cells

A549 cells were incubated with (A) brefeldin A, to inhibit endocytosis, (B) cytochalasin D, to disrupt actin polymerization, or (C) nocodazole, a microtubule-disrupting agent prior to incubation with 10 μg/ml (2.2 μM) LL-37B for 4 h. Cells were prepared for immunofluorescence as described in Materials and Methods. The right panels represent a merger of LL-37 (left panel, green), actin (A, C) or microtubules (B) (middle panels, red). Bars represent 20 μm.
Figure 13: **LL-37B does not co-localize with markers of endocytosis in A549 epithelial cells**

LL-37 does not co-localize with LAMP at 15 min (A), golgi (B), endoplasmic reticulum (C), cathepsin D (D) at 4 h. A549 cells were incubated with 10 μg/ml (2 μM) LL-37B for 15, 30 min, 1, 2 and 4 h when examining for co-localization with markers of endocytosis. Selected time points are shown. The right panels represent a merger of LL-37 (left panels, green) and the markers of the endocytic pathway (middle panels, red). Bars, 20 μm
receptor FPRL-1 in human monocytes and epithelial cell lines (Bowdish, et al. 2003, Koczulla, et al. 2003). It has been previously published that epithelial cells express FPRL-1 (Bonnans, et al. 2003, Le, et al. 2001), and indeed FPRL-1 expression at the mRNA level was confirmed by RT-PCR in the A549 epithelial cell line (Figure 14). To determine if the uptake of LL-37B was mediated by a G_i protein-coupled receptor, such as FPRL-1, cells were incubated with pertussis toxin, (100 ng/ml) for 2 h prior to the addition of 10 μg/ml LL-37B, to inactivate all G_i protein-coupled receptors. LL-37B uptake and localization to the perinuclear region was unaffected at 4 hours (Figure 15A). In addition the FPRL-1 hexapeptide agonist, WKYMVM (Dahlgren, et al. 2000), was investigated as a potential competitive inhibitor. LL-37 entry and trafficking to the perinuclear region was unaffected in the presence of 50 or 500 nM of this agonist (Fig 15B, C), comparable to the K_d of 160 nM for agonist binding to FPRL-1.

3.1.6 LL-37 induced IL-8 expression is inhibited by brefeldin A.

LL-37 has been shown to induce the production of IL-8, a neutrophil specific chemokine, in whole human blood (Scott, et al. 2002) as well as in cultured epithelial cell lines (Scott, et al. 2002, Yang, et al. 2001). To determine if endocytosis was important for the expression of IL-8, A549 epithelial cells were treated with the endocytosis inhibitor, brefeldin A, for 1 h prior to the addition of 10 μg/ml LL-37 for up to 4 h, and real-time RT-PCR was used to determine the gene expression of IL-8. There was an increase in IL-8 expression up to 2 h after incubation with LL-37 and a decrease in expression at 4 h, in the absence of brefeldin A. In contrast, when the entry of LL-37 into cells was inhibited with brefeldin A, there was no significant increase in IL-8 expression, when compared with untreated controls (Figure 16).
Figure 14: A549 epithelial cells express formyl peptide receptor like-1 (FPRL-1)

RNA was isolated from A549 epithelial cells and total RNA was used to make cDNA. Detection of FPRL-1 was done by RT-PCR resulting in a 202 bp product. (A) 100 bp ladder; (B) water control; (C) RT negative control; (D) A549 epithelial cells; (E) positive control.
Figure 15: Uptake and localization of LL-37B does not involve $G_i$ protein-coupled receptors

A549 epithelial cells were preincubated with (A) pertussis toxin (100 ng/ml, 2 h), to inhibit all $G_i$ protein-coupled receptors, before the addition of 10 $\mu$g/ml (2.2 $\mu$M) LL-37B for 4 h at 37°C. W-peptide, FPRL-1 agonist, was added at (B) 50 nM or (C) 500 nM, along with 10 $\mu$g/ml LL-37 for 4 h and 37°C. Cells were prepared for immunofluorescence as described in Methods and Materials. Right panel represent a merge of LL-37 (left panel, green) and actin (middle panel, red). Bars represent 20 $\mu$m.
Figure 16: LL-37-induced IL-8 gene expression can be inhibited by brefeldin A

A549 cells were incubated with (□) brefeldin A (1 h, 5 μg/ml) or (■) untreated prior to the addition of 10 μg/ml LL-37 (2.2 μM) for up to 4 h. Real time RT-PCR was performed using primers to human IL-8 gene and GAPDH. The fold change was normalized to GAPDH to correct for inconsistencies. A ratio of one indicates no stimulation. Results are expressed as mean ± standard error of 3 separate experiments. Student's two-tailed t-test was performed. **, p < 0.01; *, p < 0.05.
3.1.7 **LL-37B is bound by two receptors and the low affinity receptor appears to be FPRL-1**

Binding assays were performed to determine whether A549 epithelial cells have specific receptor(s) for LL-37. The resulting Scatchard analysis indicated that LL-37 was able to bind to two classes of receptors, consistent with the general conclusion for mast cells (Niyonsaba, et al. 2002). The studies presented here indicate that the A549 epithelial cell line used here, have a high affinity receptor with a dissociation constant \(K_d\) of 0.69 ± 0.28 µM (3.5 µg/ml) and a low affinity receptor with a \(K_d\) of 2.77 ± 0.37 µM (14.2 µg/ml) (Fig. 17). These \(K_d\) values were somewhat lower (i.e. affinity was higher) than the estimated values of 2.2 and 123 µM based on radioactive analysis of mast cell receptors (12). Competitive binding assays using the FPRL-1 agonist, W-peptide, showed that the binding of LL-37B to the low affinity receptor was inhibited by the presence of W-peptide, suggesting that FPRL-1 is the low affinity receptor (Fig. 18) in contrast to observations with mast cells for which the low affinity receptor was not FPRL-1.

3.1.8 **Summary**

The interaction of LL-37 with eukaryotic cells was characterized in tissue culture medium by using biotinylated LL-37 and confocal microscopy. LL-37 was actively taken up into A549 epithelial cells and eventually localized to the perinuclear region. Specific inhibitors were used to demonstrate that the uptake process was not mediated by actin but required elements normally involved in endocytosis, and that trafficking to the perinuclear region was dependent on microtubules. The expression of the chemokine IL-8, a known consequence of LL-37 treatment, was also found to be dependent upon the uptake of LL-37 by A549 epithelial cells. Binding assays also suggest that there are high affinity and low affinity receptors, of which the low affinity receptor appears to be FPRL-1.
Figure 17: LL-37B binds to two receptors on A549 epithelial cells

Scatchard plot analysis of the binding of LL-37B to A549 epithelial cells. A549 cells were preincubated with brefeldin A (5 μg/ml) for 1 h prior to the addition of 2 to 1000 μg/ml LL-37B for 15 min at 37°C. Streptavidin-HRP and TMB was added sequentially to visualize binding. Each point represents the mean of 3 individual experiments, each of which was assayed in duplicate.
Figure 18: The low affinity receptor for LL-37B appears to be formyl peptide receptor like-1 (FPRL-1)

Competitive binding studies with FPRL-1 agonist, W-peptide, were done by pre-incubating A549 cells with brefeldin A (5 μg/ml) for 1 h prior to the addition of 2 to 50 μg/ml LL-37B alone (▲) or with 10 μM W-peptide (■) for 15 min at 37°C. Streptavidin-HRP and TMB was added sequentially to visualize binding. Each point represents the mean of 3 individual experiments, each of which was assayed in duplicate.
3.2 Activities of LL-37 in lung epithelia

3.2.1 Introduction

LL-37 has been previously demonstrated to be cytotoxic towards eukaryotic cell lines at high concentrations (Johansson, et al. 1998, Oren, et al. 1999). In addition, the bovine cathelicidins, BMAP-27 and BMAP-28, which have also demonstrated to be cytotoxic, were shown to induce apoptosis in a human monocyte-cell line (Risso, et al. 1998). In this chapter, the cytotoxicity of LL-37 at higher concentration was revisited and LL-37 was tested for its cytotoxicity towards A549 epithelial cells as well as its ability to induce apoptosis. The effects of different serum conditions were examined along with individual serum components on LL-37-induced cytotoxicity. This chapter focuses on the effects of different serum conditions on LL-37’s biological properties.

3.2.2 The cytotoxicity of LL-37 on A549 lung epithelial cells is serum dependent

To investigate the cytotoxic nature of LL-37, A549 epithelial cells were incubated with 10 – 300 µg/ml LL-37 for 24 h at 37°C in DMEM in the absence of serum or with 10% FBS or pooled AB human serum. The level of lactate dehydrogenase (LDH) released by damaged cells into the supernatant was quantified as a marker of cytotoxicity (Fig. 19). With no serum supplementation, LL-37 was cytotoxic towards A549 cells at concentrations as low as 25 µg/ml LL-37 (p < 0.05). In the presence of FBS, cytotoxicity was not observed at concentrations less than 50 µg/ml LL-37 (p < 0.01). However, in human serum, cytotoxicity towards A549 cells was not observed at concentrations less than 200 µg/ml LL-37 (p < 0.01). These findings suggest that there are one or more serum components in HS that modify the cytotoxic effects of LL-37.
Figure 19: Cytotoxic effects of LL-37 in different serum conditions as measured by lactate dehydrogenase (LDH) release

A549 epithelial cells were stimulated with 10 to 300 µg/ml LL-37 for 24 h in DMEM supplemented with 10% HS (□), 10% FBS (■) or no serum (□). The levels of LDH in the supernatant were measured as described in Methods and Materials. 100% LDH release was the amount of LDH released into the supernatant when 1% Triton X-100 was incubated with cells. 0% LDH release was taken as the amount of LDH in media alone. Results are expressed as mean ± standard error of 3 separate experiments. Student’s two-tailed t-test was performed. **, p < 0.01; *, p < 0.05.
3.2.3 **Localization of LL-37B in lung epithelial cells is serum-dependent**

The cellular localization of LL-37B has been demonstrated to be perinuclear in A549 epithelial cells in the presence of FBS (Fig. 20). To determine effects of serum on the localization of LL-37B, A549 epithelial cells were incubated with 10 µg/ml LL-37B in the presence of DMEM along with 10% HS or no serum. In the absence of serum, LL-37B localized to the nucleus of approximately 20% of the cells and therefore, may account for the cytotoxicity of LL-37 as measured by the LDH assay. In the presence of HS, LL-37B was not observed to be present within cells. Interestingly, different results were observed for the two batches of HS that were used. One batch of HS demonstrated that, although LL-37B did not localize within cells, aggregates were formed that were cell associated. For the other batch of HS, LL-37B did not localize within cells and no cell-associated aggregates were seen.

3.2.4 **LL-37 induces apoptosis in A549 epithelial cells**

One of the trademarks of apoptosis is the cleavage of genomic DNA, which can be assessed using the TUNEL assay. Free 3'-OH DNA ends are created when caspase-activated DNAse generates 180 bp DNA fragments from high molecular weight DNA fragments. Fluorescent labels can then be incorporated by terminal deoxynucleotidyl transferase (TdT), an enzyme that catalyzes the repetitive addition of mononucleotides from dNTP to the terminal 3'-OH of a DNA strand. The fluorescent labels that are incorporated can be directly quantified by fluorescence microscopy. LL-37 was tested for its ability to induce apoptosis in A549 epithelial cells in different serum conditions. As demonstrated in Figure 21A, LL-37 caused an increase in TUNEL-positive cells in a dose-dependent manner at concentrations equal to or greater than 50 µg/ml in FBS. In the presence of human serum, LL-37 did not induce A549 cells to become TUNEL-positive even at concentrations as high as 100 µg/ml. TUNEL-positive cells were quantified by counting the ratio of TUNEL-positive cells to total cells in 3 fields of view in 3
Figure 20: Localization of LL-37B differs in the presence of various types of serum

A549 epithelial cells were incubated at 37°C with 10 µg/ml (2.2 µM) LL-37B for 4 h in DMEM with 10% human serum (A), 10% fetal bovine serum (B) and no serum (C). Cells were prepared for immunofluorescence as described in Methods and Materials. Right panels represent a merge of LL-37B (left panels, green) and actin (middle panels, red). Bars represent 20 µm.
independent experiments (Figure 21B). At a concentration of 50 µg/ml LL-37 in the presence of FBS, 20% of the cells were TUNEL-positive and the number of TUNEL-positive cells increased to 45% at concentration of 100 µg/ml LL-37.

LL-37 induced apoptosis in FBS was confirmed by examining the activation of caspase-3, a common effector for all apoptotic pathways. The effect of LL-37 on pro-caspase-3 in FBS was examined by Western blot analysis and is shown in Figure 22. The Western blot shown is a representative of 3 independent experiments and quantification involved an averaging of 3 experiments using the housekeeping gene, actin, to correct for loading inconsistencies. The native caspase-3 is 36 kDa and in inducing apoptosis through the extrinsic or intrinsic pathways, it is cleaved at Asp-175 to form two fragments, 17 and 19 kDa. A decrease in the amount of the native caspase-3 suggests that it is undergoing activation. LL-37 treatment caused a decrease in levels of pro-caspase-3 in FBS starting at concentrations of 25 µg/ml.

3.2.5 Effects of high-density lipoproteins and apolipoprotein A-1 on LL-37-induced cytotoxicity

The major component of high-density lipoproteins (HDL) is apolipoprotein A-1 (Apo A-1) (Sorci-Thomas, et al. 2002). HDL and ApoA-1 confer protection against atherosclerosis by participating in the reverse cholesterol transport from peripheral cells to the liver. Apolipoprotein A-1 binds LL-37 in human plasma, potentially working as a scavenger of LL-37 and therefore may be involved in cathelicidin regulation (Sorensen, et al. 1999, Wang, et al. 1998). Concentration of HDL varies in human serum between 0.94 – 1.99 mg/ml (Schumaker, et al. 1986). Since the presence of apolipoprotein A-1 has been demonstrated to inhibit antimicrobial function, I hypothesized that it might also affect cytotoxicity. To determine if apolipoprotein A-1 or HDL were involved in reducing the cytotoxic effects of LL-37, A549 epithelial cells were incubated with either HDL or Apo A-1 in the presence or absence of fetal bovine serum for 24 h. The levels of lactate dehydrogenase
A549 epithelial cells were incubated with 10 to 100 μg/ml LL-37 or media alone in the presence of 10% FBS or 10% HS for 24 h. TUNEL assays were performed as described in Methods and Materials. (A) The amount of TUNEL-positive cells cultured in 10% FBS (○) and 10% HS (■) were quantitated by counting 3 fields of view. Results are expressed as mean ± standard error for 3 separate experiments. Student’s two tailed t-test was performed. **, p < 0.01. (B) The nucleus of TUNEL positive cells were stained green (denoted by white arrows). All nuclei were stained blue with DAPI. Bars represent 50 mm.
0 µg/ml LL-37
10 µg/ml LL-37
25 µg/ml LL-37
50 µg/ml LL-37
75 µg/ml LL-37
100 µg/ml LL-37
Figure 22: Effects of LL-37 on caspase-3 levels in A549 cells as measured by Western blot analysis

A549 epithelial cells were stimulated with 10 to 100 μg/ml LL-37 or media alone for 24 h at 37°C. (A) Total cell lysates were prepared for Western blotting and the membrane was probed for caspase-3 and actin as described in Methods and Materials. (B) The intensities of the Western blot were measured and normalized to actin to correct for inconsistencies in loading. The stimulated protein levels relative to unstimulated was calculated as the mean ± standard error for 3 separate experiments. The bar graph shows that ratio of protein expression of LL-37 stimulated/unstimulated cells. A ratio of one indicates no stimulation. Student’s two tailed t-test was performed. **, p < 0.01; *, p < 0.05.
(LDH) released by damaged cells into the supernatant were measured. In the presence of 10% fetal bovine serum, Apo A-1 was unable to block the cytotoxic effects of 50 μg/ml LL-37. However, 650 μg/ml HDL completely blocked the cytotoxic effects of 50 μg/ml LL-37 and the blocking occurred in a dose-dependent fashion although concentrations of HDLs greater than 3250 μg/ml appeared to be themselves toxic (Figure 23). The same trend was observed in the absence of serum where Apo A-1 was unable to block or reduce the cytotoxic effects of LL-37 but the presence of HDL could (Figure 24).

3.2.6 Summary

LL-37 was found to be cytotoxic towards A549 epithelial cells in the absence of serum and in the presence of FBS but not in the presence of HS. In the presence of FBS, LL-37 induced apoptosis of epithelial cells as measured by the TUNEL assay. Western blot analysis for procaspase-3 confirmed that LL-37 induced apoptosis. High density lipoproteins but not apolipoprotein A-1 present in human serum were able to block the cytotoxicity of LL-37, suggesting that apolipoprotein A-1 was required to be in a native conformation for binding LL-37 to prevent LL-37-induced cytotoxicity or that there was a role for lipids in the protection of cells from high concentration of LL-37.

In addition, the localization of LL-37B was dependent on the type of serum present during incubation. LL-37B was not present within cells in the presence of HS but was found in the nucleus when no serum was present. In FBS, as previously shown, LL-37B was seen in vesicular structures in the cytoplasm.
Figure 23: High density lipoprotein (HDL) but not apolipoprotein A-I (Apo A-I) is able to protect against LL-37-induced cytotoxicity in the presence of 10% fetal bovine serum

A549 epithelial cells were incubated with 50 µg/ml LL-37 alone and along with increasing concentrations of (A) Apo A-I or (B) HDL in the presence of 10% fetal bovine serum. The levels of LDH in the supernatant were measured as described in Methods and Materials. 100% LDH release was the amount of LDH released into the supernatant when 1% Triton X-100 was incubated with cells. 0% LDH release was taken as the amount of LDH in media alone. Results are expressed as mean of 2 separate experiments. Student’s two tailed t-test was performed. **, p < 0.01; *, p < 0.05.
Figure 24: High density lipoprotein (HDL) but not apolipoprotein A-1 (Apo A-1) is able to protect against LL-37 induced cytotoxicity in the absence of serum.

A549 epithelial cells were incubated with 50 µg/ml LL-37 alone and along with increasing amounts of (A) Apo A-1 or (B) HDL in the absence of serum. The levels of LDH in the supernatant were measured as described in Methods and Materials. 100% LDH release was the amount of LDH released into the supernatant when 1% Triton X-100 were incubated with cells. 0% LDH release was taken as the amount of LDH in media alone. Results are expressed as mean of 2 separate experiments. Student’s two tailed t-test was performed. **, p < 0.01.
4. DISCUSSION

The mechanism of interaction of cationic host defense peptides with human cells has not been well characterized to date. There have been a number of studies looking at the uptake by eukaryotic cells of several peptides belonging to different structural classes (Chan, et al. 1998, Drin, et al. 2003, Stauber, et al. 1998, Suzuki, et al. 2002, Takeshima, et al. 2003). In this thesis, I examined the interaction of the human cathelicidin peptide, LL-37, with the human lung epithelial cell line, A549. Using biotinylated LL-37 (LL-37B) together with confocal microscopy, I was able to visualize the uptake of this peptide by epithelial cells.

I first demonstrated that the labeling of LL-37 with biotin did not alter the structural or biological behavior of this peptide. Both LL-37B and LL-37 adopted similar α-helical secondary structures upon membrane binding as assessed by CD spectrometry consistent with published Fourier-transform infrared spectroscopy studies for LL-37 (Oren, et al. 1999). Also, similar patterns of A549 cell gene expression were observed in response to both LL-37 and LL-37B with only 14 of 14,000 genes differing in expression levels by more than 4-fold. In addition, both peptides stimulated similar levels of IL-8 expression and LDH release. These results indicate that LL-37B provided an excellent model for visualizing the interactions between epithelial cells and LL-37.

The images presented here demonstrate that, in the presence of FBS, LL-37B was actively taken up into A549 cells. Over a period of 4 hours, at concentrations that are achievable in vivo, the peptide first became rapidly dispersed throughout the cytoplasm in small vesicles and then trafficked to the perinuclear region. The localization of LL-37B to the perinuclear region was confirmed using unlabelled LL-37 and visualized using specific antibodies. The vesicular structures seen at all time points were suggestive of an endocytic process by which LL-37 was transported into the cell. Similar observations have been made with SynB3 and SynB5, synthetic
linear analogues of the β-hairpin structured peptide protegrin-1 (Drin, et al. 2003). Using the K562 cell line and TAMRA-labeled peptide, it was demonstrated that the uptake of SynB3 and SynB5 was inhibited at low temperature, but at 37°C these peptides became localized to the perinuclear region in a punctate pattern consistent with, but not proving, a location within endocytic vesicles (Drin, et al. 2003). PR-39, a proline-arginine-rich peptide, also enters eukaryotic cells and localizes throughout the cytoplasm, binding to several cytoplasmic proteins (Chan, et al. 1998). Recently, it was shown that LL-37 can bind extracellular DNA, and that the LL-37-DNA complex is taken up through caveolae-independent membrane raft endocytosis and cell surface proteoglycans and the resulting complex is found in vesicular structures at the cell membrane and throughout the cytoplasm (Sandgren, et al. 2004).

Since cationic host defense peptides are reminiscent of peptides with nuclear localization signals, we had anticipated that LL-37 would migrate to the nucleus, something that was observed in these studies only when higher, somewhat toxic concentrations were used. The observation of nuclear localization can indeed be an artifact as, for example, demonstrated for the cationic arginine-rich Tat peptide from HIV, which had been shown to localize to the nucleus via protein transduction (Suzuki, et al. 2002). Recently it has been shown that cell fixation promotes penetration of the nucleus by Tat due to the strong cationic nature of this peptide (Drin, et al. 2003, Richard, et al. 2003). At 37°C, such cell-penetrating peptides, including Tat, are apparently not localized to the nucleus, as previously proposed, but instead distribute throughout the cytoplasm in a punctate pattern in epithelial cells (Richard, et al. 2003). As observed here for LL-37, endocytosis was decreased in cells at low temperatures and at 4°C there was no intracellular accumulation of peptide with the majority of the peptide being seen around the periphery of cells.

At higher concentrations, LL-37B was taken up into the nucleus of cells and it was observed that in these cells, staining of F-actin by phalloidin was lost, presumably reflecting the
binding of LL-37 to F-actin, as seen previously (Weiner, et al. 2002). At lower concentrations, when cells were not permeabilized for immunohistochemical analysis, cells treated with LL-37 still stained for actin. This indicates that LL-37 was itself able to permeabilize the eukaryotic membrane. Similarly, it has been demonstrated that Tat can permeate cell membranes without disturbing membrane integrity (Stauber, et al. 1998, Suzuki, et al. 2002). In contrast, the host defense peptide PR-39 has been shown to enter mesenchymal cells without permeabilizing the plasma membrane (Chan, et al. 1998).

Specific inhibitors were utilized to explore the possibility that LL-37 was taken up by an endocytic process. The uptake of LL-37 into A549 epithelial cells was inhibited when endocytosis was disrupted with brefeldin A, however, the disruption of actin polymerization by cytochalasin D did not block the uptake and perinuclear localization of the peptide. Although actin was not required for uptake of LL-37, it seemed possible that other elements of endocytosis are required. LL-37 trafficking to the perinuclear region was indeed dependent on an intact network of microtubules, since the microtubule-disrupting agent, nocodazole, prevented cellular accumulation of the peptide. Cellular markers involved in endocytic transport such as LAMP-1, golgin-97, calnexin, and cathepsin D were also examined but did not co-localize with LL-37B at the time points studied. Since LL-37 is a highly charged molecule, it is possible that this altered the endosomal/lysosomal compartment and therefore, prevented the association of some of the markers normally involved in endocytosis. Alternatively, LL-37 might have been co-localizing with components of the endocytic pathway at different time points than those used in this study. In contrast to our LL-37 observations, it has been suggested that, once internalized, linearized protegrin-I peptides follow a conventional endocytic pathway towards late endosomes and lysosomes (Drin, et al. 2003). In another published study, LL-37-DNA complexes were shown to co-localize with cholera toxin subunit B, a marker for lipid rafts, in vesicular structures but no
co-localization was seen with caveolin, a well-established marker of caveolae at the site of endocytosis (Sandgren, et al. 2004).

Since LL-37 is internalized into epithelial cells, we wished to characterize the binding of LL-37 to the surface to cells that would precede uptake. The binding of LL-37B to A549 epithelial cells was demonstrated to involve two receptors, and the low affinity binding receptor appeared to be FPRL-1. These observations are consistent with other studies of LL-37 interaction with cells indicating multiple receptors on the cell surface (Bowdish, et al. 2003, Niyonsaba, et al. 2002, Tjabringa, et al. 2003, Yang, et al. 2000). The binding was concentration dependent and saturable. The receptor densities were estimated to be more than $10^7$ binding sites/cell. I consider however that these numbers are overestimates caused by the requirement to use an amplification step to assess LL-37B, as well as quenching of fluorescence due to the presence of cells.

I was able to demonstrate that the FPRL-1 agonist, WKYMVM, inhibited the binding of LL-37B to the low affinity receptor, favoring the concept that the $G_i$ protein-coupled receptor FPRL-1 was this low affinity receptor. Nevertheless it was demonstrated that pertussis toxin did not affect uptake or localization, and consistent with this, it has been previously shown that FPRL-1 is not a functional receptor for mast cell chemotaxis (Niyonsaba, et al. 2002), nor for monocyte or epithelial cell signaling (Bowdish, et al. 2003, Tjabringa, et al. 2003). Therefore since the uptake of LL-37B was further unaffected by competition with the FPRL-1 agonist, it seems likely that the high affinity receptor was responsible for uptake.

Many known antimicrobial peptides are cytotoxic towards a range of microorganisms but not eukaryotic cells. It has been published that the bovine cathelicidins, BMAP-27 and BMAP-28, are known to exhibit cytotoxicity towards eukaryotic cells as well as microbes and that the cytotoxicity is mediated by their hydrophobic C-terminal tail (Risso, et al. 1998). However, LL-37 is also known to exhibit cytotoxic activity towards eukaryotic cells, although since LL-37 doesn’t have a hydrophobic tail, the mechanism of cytotoxicity is most likely different than for
the bovine cathelicidins (Johansson, et al. 1998). We were able to demonstrate that LL-37 was more cytotoxic in the absence of serum than in the presence of 10% human serum, with 10% fetal bovine serum permitting an intermediate effect. Other groups have also observed these findings along with the fact that the antibacterial activity of LL-37 is also reduced in the presence of human serum (Johansson, et al. 1998, Sorensen, et al. 1999, Wang, et al. 1998).

Since it has been suggested that ApoA-1 acts as the main LL-37 binding protein ($K_d$ of 0.6-2.4 μM) in human serum, we examined the ability of ApoA-1 as well as HDL to inhibit the cytotoxic effects of LL-37. Although ApoA-1 did not prevent the cytotoxic effects of LL-37, HDL inhibited LL-37 induced cytotoxicity at relevant concentrations both in the presence of 10% fetal bovine serum and in the absence of serum. This indicates that even though LL-37 has been shown to bind ApoA-1, it may need to be in its proper lipid binding conformation to prevent the cytotoxic effects of LL-37. In its lipid bound state, as part of HDL, ApoA-1 consist of four molecules with lipid binding domains on the inside and upon delipidation, the ApoA-1 structure collapses (Wang 2002). Interestingly, in addition to its role in the reverse cholesterol transport pathway, ApoA-1 has anti-inflammatory function including protection against endotoxin, binds and inhibits polymerization of complement factor C9 and inhibits IgG-induced neutrophil activation (Furlaneto, et al. 2002, Hamilton, et al. 1993, Levine, et al. 1993). Therefore, the binding of LL-37 to ApoA-1/HDL may be a way to regulate and contain inflammation. Analogously, defensins (HNP-1) are bound by activated α2-microglobulin (Panyutich, et al. 1991).

The cytotoxicity of BMAP-27 and BMAP-28 is due to membrane permeabilization leading to programmed cell death (Risso, et al. 1998). More recently, BMAP-28 has been shown to affect mitochondrial membrane permeability causing mitochondrial depolarization and cytochrome c release leading to cell death (Risso, et al. 2002). LL-37 has also been shown to apparently induce apoptosis when introduced intratracheally into BALB/c mice, as assessed by
the TUNEL assay (D.J. Davidson, unpublished results). TUNEL assay was used to determine if LL-37 caused apoptosis in A549 epithelial cells. There was a dose-dependent increase in TUNEL positive cells starting at 50 µg/ml LL-37 in the presence of fetal bovine serum but interestingly, cells did not become TUNEL positive up to 100 µg/ml LL-37 in the presence of human serum. This indicates that there are component(s) present in human serum, possible ApoA-1 or HDL that modified the effect of LL-37 on eukaryotic cells. To confirm that LL-37 did induce apoptosis through the well-known caspase pathways, the effect of LL-37 on pro-caspase-3 was examined in the presence of fetal bovine serum. Although there was a decrease in the amount of pro-caspase-3, cleaved caspase-3 was not detected. Caspase-3 activation occurs in two steps: an initial cleavage event followed by autoactivation that is controlled by the self-catalytic activity of caspase. High levels of BCL-2 prevent the subsequent caspase autoactivation leading to only partial cleavage of caspase-3, suggesting that it may be hard to detect the cleaved form of caspase-3. For full caspase activation, pro-apoptotic mitochondrial protein (SMAC/DIABLO) is required to block the effects of inhibitors of apoptosis proteins (IAP). This phenomenon is seen with granzyme B mediated cell death, which can cause direct activation of caspase-3 (Goping, et al. 2003, Sutton, et al. 2003). Thus it is not entirely clear at which point LL-37 induces caspase-3 activation.

Interestingly, the uptake and localization of LL-37B differed with the type of serum present. As mentioned previously, in the presence of FBS, LL-37B localized to the perinuclear region. In the absence of serum, LL-37B localized to the nucleus of what appeared to be dividing cells; this is similar to the observation made when A549 epithelial cells were treated with 50 µg/ml LL-37B (Figure 10). However, in the presence of human serum, LL-37B was not detected inside of A549 epithelial cells. Two different observations were made with different lots of human serum. For one lot, LL-37B was detected on the outside of cells in aggregates that were cell associated, while in the other lot of human serum there were no observed cell-
associated aggregates of LL-37B, and LL-37B was not detected anywhere in the field of view. These observations lead to the hypothesis that HDL was responsible for the aggregation of LL-37 and that different lots of human serum may have different levels of lipoproteins.

Previously, it has been published LL-37 and other cationic peptides are produced by respiratory epithelium and can be detected in airway surface fluid at basal levels and induced to be secreted upon detection of infection and inflammation. The unprocessed form of LL-37, hCAP-18, has also been detected in airway surface fluid. Although high concentrations of LL-37 have been detected in psoriasis and in lung lavage, it is likely that in serum, LL-37 is regulated by binding to serum components, such as HDL, to regulate activity. LL-37 and other cationic peptides are found at low concentrations in normal tissues, and several peptides can be found in a single tissue suggesting that they may have complementary activity. It is generally believe that these peptides can synergize with each other and with other components present in the tissue such as lactoferrin and lysozyme, increasing its antimicrobial activity. In summary, I have shown that LL-37 can interact with and cause a range of responses from human epithelial cells and that LL-37 mediates the host response through cell surface receptors. Since differences in the biological activity of LL-37 are seen with different sera types, physiological relevance as well as serum components need to be further explored.
REFERENCES


of structural requirements for their antimicrobial and cell lytic activities. J Biol Chem. 271:28375-28381.


