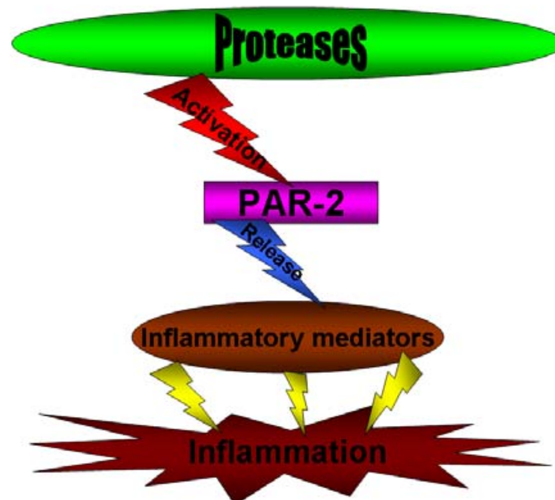
King crab proteases, as stimulators of an inflammatory pathway in human airway epithelial cells



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The effort herein is dedicated to my loving parents for their prayers, sacrifices, unconditional, endless support and encouragement.

I am honoured to have you as my parents!

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Abbreviations

A549	human pulmonary epithelial cells
APC	Antigen Presenting Cells
b	slope
CO_2	Carbon dioxide
$CaCl_2$	Calcium chloride
dH_2O	Distilled water
DMEM/F12	Dulbecco's Modified Eagle Medium F12
DMSO	Dimethyl Sulfoxide
dsRNA	Double stranded Ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
F VIIa	Clotting factor VIIa
F Xa	Clotting factor Xa
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HAT	Human airway trypsin-like protease
IgE	Immunoglobulin E
IL-6	Interleukin-6
IL-8	Interleukin-8
Ln	Log natural
MCP-1	Monocyte chemotactic protein-1
miRNA	Micro Ribonucleic acid
MMP	Matrix metalloproteinase
NaOH	Sodium hydro oxide
ND	Not determined
Opti-MEM	Reduced serum medium
P. Aeruginosa	Pseudomonas aeruginosa
PAR	Protease activated receptor
PARs	Protease activated receptors
PGE2	prostaglandin E2
RNAi	Ribonucleic acid interference
rpm	Revolutions per minute
S.D	Standard deviation
siRNA	small interfering Ribonucleic acid
Streptavidin-HRP	Horse radish peroxidase labeled Streptavidin
T_2	Doubling time
TNF alpha	Tumor necrosis factor-alpha

Summary

There have been valuable researches regarding health problems at seafood processing areas and with seafood industry workers. In the past few years airway symptoms have been reported in the seafood industry workers. This study was carried out on the background that airway symptoms are generated in king crab industry workers.

In these *in vitro* studies, we focus on moricrase (enzyme mixture from hapatopancreas of king crab) as an activator of inflammatory responses in airway cells. King crab industry workers are highly exposed to bioaerosoles containing enzymes as well as other bioactive agents. These bioaerosoles are produced during industrial processing of king crab. Taking under consideration that serine proteases, such as trypsin, are stimulator of interlukin-8 (IL-8). We investigate the role of protease activated receptor 2 (PAR-2) in the inflammation of human pulmonary epithelial cells (A549). PAR-2 is considered as an important factor under the pathological situations in human airways. Human airway epithelial cells were exposed to moricrase and we observed that moricrase induces stimulation of IL-8.

In the dose-response experiments moricrase has shown stimulation of IL-8 in A549 cells. In six hours incubation with different concentrations of moricrase, A549 cells have shown maximum levels of IL-8 with 0.03325 µg/ml of moricrase. In our time response data, the cells have shown a gradual increase in IL-8 production and a maximum production of IL-8 (more than 90% above of basal level) was found after 24 hours incubation.

Finally, it has been found that after the stimulation of A549 cells with moricrase, the release of IL-8 is mediated through the activation of PAR-2 by using specific small interfering RNAs (siRNAs). Although the release of IL-8 after stimulation with moricrase was lower than the IL-8 release after stimulation with spotted mackerel trypsin. Our data confirms the involvement of PAR-2 as by using PAR-2 siRNAs, the production of IL-8 was reduced 85.5%. With respect to our findings we can suggest that moricrase, via activation of PAR-2, might produce inflammation or influence the inflammatory process in human airways if inhaled in a significant amount.

Aim of the study

King crab industry workers are highly exposed to bioaerosoles. These tiny water droplets may contain some proteases that may have effects on the human respiratory system. King crab industry workers have shown airway symptoms. Our study was aimed to find out whether moricrase (a mixture of collageno and fibrinolytic proteinases from *Paralithodes camchatica* (king crab) hepatopancreas) stimulates the inflammatory mediator (IL-8) in human pulmonary epithelial (A549) cells and if so, to characterize the time and dose dependency of such stimulation. Moreover, to find out if moricrase stimulates IL-8 via the PAR-2 mediated pathway.

1. Introduction

1.1. Human Airway.

Lungs are the organs of respiration in humans. Many foreign substances are inhaled daily, but the lungs have mechanisms that usually prevent people from making unwanted immune responses to these materials. Sometimes, however, immune responses are generated to these substances, resulting in allergic responses and airway symptoms (Hammad *et al.*, 2009).

Breathing in certain dusts, gases, fumes and vapors within the workplace can cause various airway symptoms from upper as well as lower respiratory tract, like shortness of breath, sneezing, running nose, etc.

Airway symptoms have been reported in the seafood industries (Gaddie *et al.*, 1980). The employees in the seafood industry are exposed to a variety of factors that may cause airway symptoms (Bang *et al.*, 2005). Water is used extensively in many processes of the seafood industry and the production area contains large amount of moisture. Production machines produce wet aerosols that spread in the breathing zone of the workers. Workers of seafood industry having increased prevalence of airway symptoms compared to control population as the bioaerosols may contain biological materials. The moist environment may also facilitate the growth of microorganisms.

1.1.1. Types of respiratory cells

More than 40 different types of respiratory tract cells have been identified. The five major cell populations of the lung include type 1 and type 2 alveolar epithelial cells, capillary endothelial cells, alveolar macrophages and interstitial cells. These cells form

the basic structure underlying gas exchange in the lung. In lung cells our interest is directed to the epithelial cells as these are the cells which can be exposed to the xenobiotics directly.

1.1.2. Alveolar type two epithelial cells

These are cuboidal cells that cover 7% of alveolar surface and represent 16% of total cell population. Type two cells are able to differentiate into type 1 cell and replace damaged cells.(Dart, 2004)

Epithelial cells protect underlying tissue from mechanical injury, harmful chemicals and pathogens and excessive water loss. Sensory stimuli are detected by specialized epithelial cells. Specialized epithelial tissue containing sensory nerve endings are found in the airway. Simple epithelium promotes the diffusion of gases, liquids and nutrients. Because they form such a thin lining, they are ideal for the diffusion of gases (e.g. walls of capillaries and lungs). [http://en.wikipedia.org/wiki/Epithelium]

1.2. Airway epithelial cells and our study

Inhalation of bioaerosols can affect the airway epithelium and can cause inflammation in lower and upper respiratory tract by the production and secretion of cytokines. Previous research explored that in the seafood industry, production workers inhale aerosols containing biological material (Bang *et al.*, 2005; Jeebhay *et al.*, 2005). The origin of biological materials can either be a raw material itself or the environmental microbiological organisms.

Processing of crab, shrimps, and clam is associated with increased risk of occupational asthma (Gaddie *et al.*, 1980; Desjardins *et al.*, 1995; Jeebhay *et al.*, 2001). Production workers in the salmon industry has shown enhanced occurrence of symptoms from the airways (Douglas *et al.*, 1995; Bang *et al.*, 2005). Allergic diseases associated with

occupational exposure to crab is well characterized, whereas for other seafood agents the evidence is somewhat limited (Jeebhay *et al.*, 2001).

1.2.1. Human alveolar epithelial cells (A549 cells)

Human alveolar type 2 epithelial cells collected in 1972 from 58 years old man infected with lung carcinoma. A549 cells showed adherent growth and they are extensively used in several experiments for the investigations of biological system.

1.3. Protease-activated receptors (PARs)

Protease-activated receptors (PARs) are a subfamily of G protein-coupled receptors that are activated by cleavage of certain part of their N terminal extra cellular domain. They are expressed throughout the whole body including lungs (Macfarlane *et al.*, 2001).

Proteases are widely distributed in biological materials, although good methods for quantification in bioaerosol samples are not yet available. Recent reports indicate the importance of serine proteases for the induction of cytokine production following exposure to molds (Borger *et al.*, 1999; Kauffman *et al.*, 2000), cockroach extracts (Bhat *et al.*, 2003) and mites (King *et al.*, 1998; Tomee *et al.*, 1998). This protease induced cytokine productions are mainly mediated by activation of protease-activated receptor (PAR)-2 (Sun *et al.*, 2001; Asokananthan *et al.*, 2002b; Page *et al.*, 2003; Hong *et al.*, 2004).

PAR activity is modulated by airway proteases of endogenous and exogenous origin, which can either activate or disable the receptors. The regulation of PAR activity by proteases is important under pathological conditions when the activity of proteases is increased (Sokolova and Reiser, 2007). Moreover, various inflammatory mediators, such as cytokines, growth factors, or prostanoids, alter the PAR expression level.

Elevated PAR levels are observed in various lung disorders, including asthma (Knight *et al.*, 2001).

1.3.1. Classification of Protease activated receptors (PARs)

PARs are classified into four known types PAR1, PAR2, PAR3, and PAR4. These receptors are members of the seven transmembrane G-protein-coupled receptor super family.

1.3.2. Activation of PARs

PAR1-PAR4 has an activation site that is recognized by specific proteases, and its cleavage results in formation of a new *N*-terminus, which serves as a tethered ligand (Macfarlane *et al.*, 2001). *Fig. 1.1* is a schematic diagram for PARs activation mechanism by proteolysis. The receptor (depicted on the left side) undergoes transformed by cleavage through a specific protease. The proteolytic activity generates the new *N*-terminus. Binding of this new tethered ligand to the extracellular loop 2, as shown on the right side, causes receptor activation and triggering of cellular responses. Some of the activating proteases, which are active in the respiratory tract, are also indicated besides the cleavage site in *Fig. 1.1*.

Previously activation of PARs was ascribed exclusively to the family of serine proteases, like thrombin and trypsin but recent findings have shown the activation of PARs from matrix metalloproteinase (MMP), namely MMP-1 (Boire *et al.*, 2005; Goerge *et al.*, 2006).

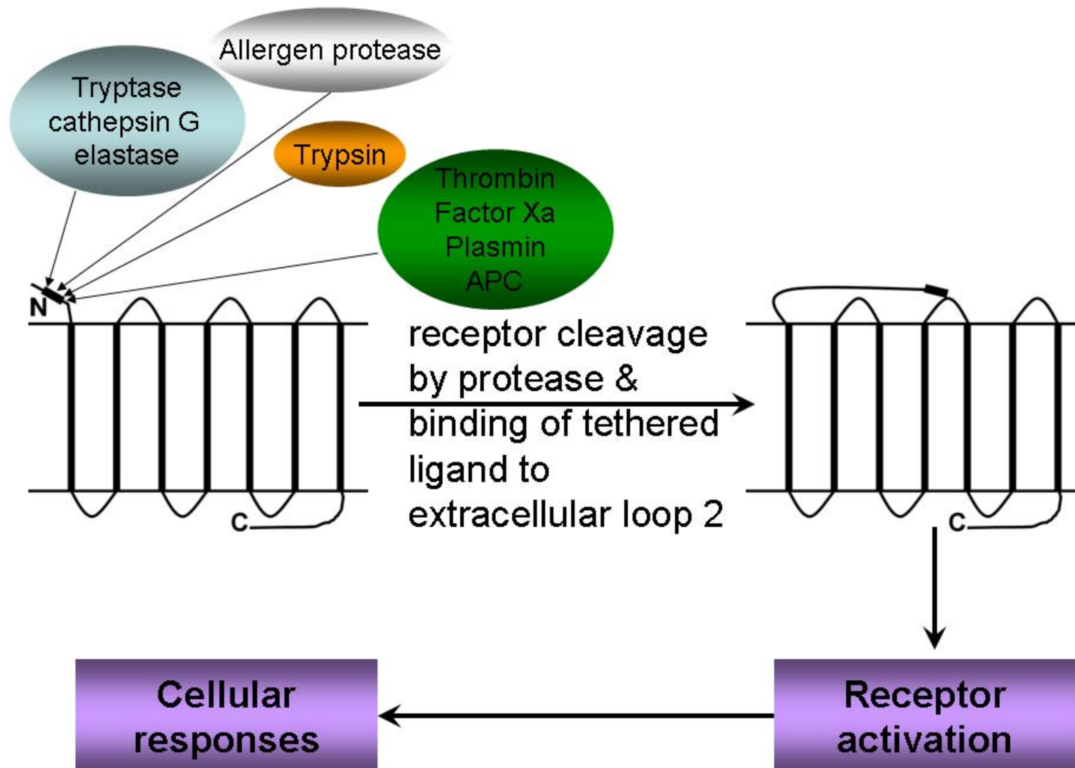

Fig. 1.1 *Mechanism of activation of protease activated receptors. The receptor consist of an extracellular N-terminus (N), 7 transmembrane domains and an intracellular C-terminus (C). Proteases of exogenous origin (from airborne allergens) and endogenous origin (belonging to the coagulation system, released from inflammatory cells, extrapancreatic trypsin) cleave the N-terminal part of PARs and unmask the tethered ligand (shown as a black box). Binding of this tethered ligand to the second extracellular loop of the receptor results in receptor activation and induction of cell signaling. Modified from.* (Sokolova and Reiser, 2007).

1.3.4. PAR-2 in human airways

The PARs are present in different tissues and cells in the mammalian body. PAR-1 and PAR-2 are present in epithelial and smooth muscle cells of respiratory system (Cocks and Moffatt, 2001; Ossovskaya and Bunnett, 2004). PAR-2 is considered as pro-

inflammatory in the respiratory system, and in asthmatic patients have found an up regulation of PAR-2 in the respiratory epithelium(Knight *et al.*, 2001).

In human alveolar epithelial cells (A549), activation of PAR-2 induces enhancement of expression and release of important inflammatory mediators such as IL-6, IL-8, and prostaglandin E2 (Asokananthan *et al.*, 2002a). matrix-metalloproteinase-9 (MMP-9) (Vliagoftis et al., 2000) and granulocyte macrophage-colony stimulating factor (GM-CSF) (Vliagoftis *et al.*, 2001).

There is still conflicting data concerning the role of PAR-2 in airway inflammatory response. Protective anti-inflammatory effects of PAR-2 activation in mice were documented (Moffatt *et al.*, 2002).

1.3.5. PAR-2 Activators.

All PARs activate with a similar mechanism. Lung epithelium is a source of potent PAR-2 activators.Epithelial derived trypsin and human airway trypsin-like protease (HAT) are endogenous activators of PAR-2 (Ostrowska *et al.*, 2007). Other endogenous proteases which possess the ability to modulate PAR activity originate from hematopoietic cells (mast cell tryptase, neutrophil elastase, cathepsin G and proteinase 3). Tryptase is released from activated mast cells in airways during allergen challenge. In the lung epithelial cells, PAR-2 can also be activated by exogenous proteinases including house dust mite allergens (Kawabata and Kawao, 2005). Serine proteases like trypsin and chymotrysin are known agonists for PAR-2.

1.3.6. Role of PAR-2 in airway inflammation

PAR-2 is present in the smooth muscle cells and, upon activation, can cause broncoconstriction via activation in smooth muscles. In the human alveolar epithelial cells , activation of PAR-2 induces release of several mediators of inflammation.

PAR-2 also mediate smooth muscle proliferation, which in addition to the pro-inflammatory roles of PAR-2 in the epithelium, may contribute to development of airway symptoms. PAR-2 can cause inflammation upon activation in human airways as shown in *Fig.1.2*. PAR-2 may also contribute to a protective effect by the activation of prostanoid (PGE_2) causing a prostanoid-dependent smooth muscle relaxation. The significance of this protective effect is still unclear.

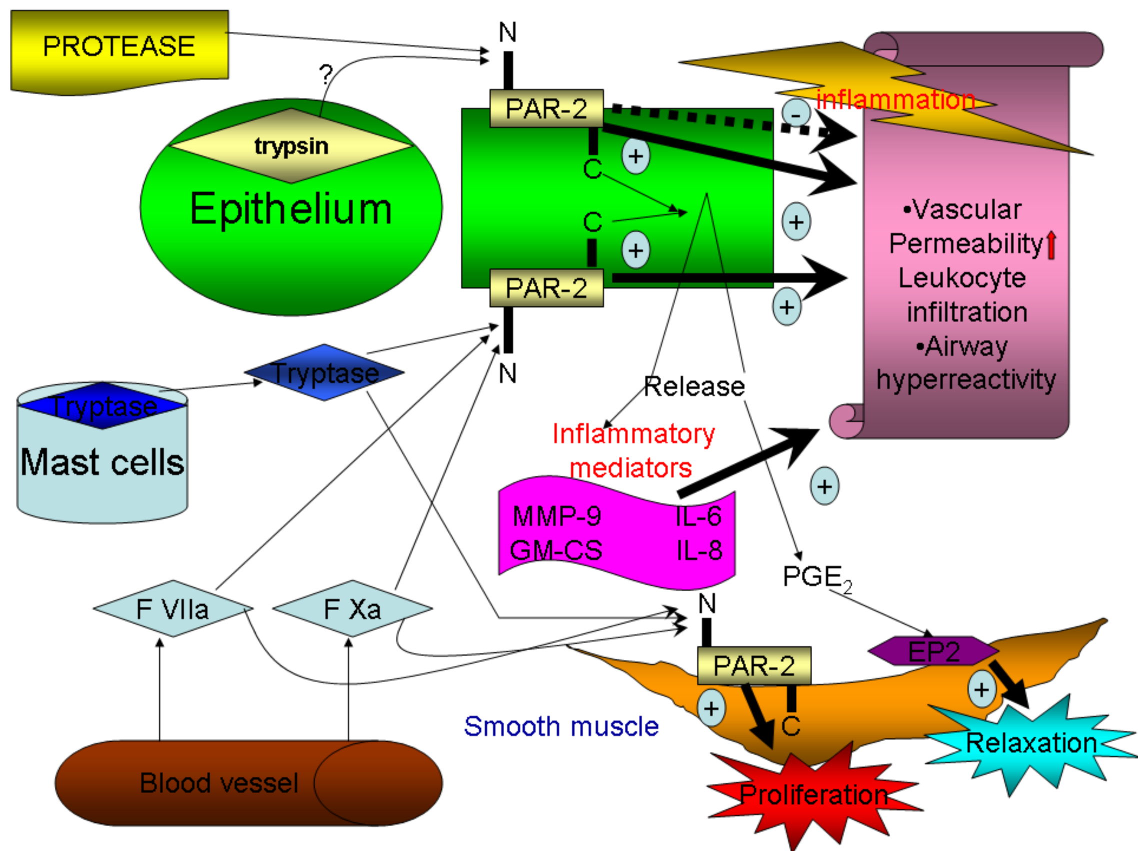

Fig.1.2. *Possible roles of PAR-2 in the respiratory system. Tryptase from mast cells, factors VIIa and Xa from blood vessels, and possibly trypsin from the adjacent epithelial cells activate PAR-2 in the epithelium, which in turn causes release of various inflammatory mediators and promotes inflammation. Crab proteases like trypsin and chymotrypsin could be exogenous agonists for PAR-2. F VIIa and F Xa, coagulation factors VIIa and Xa, respectively; PGE2, prostaglandin E2; EP2, prostaglandin EP2 receptor. Modified from (Kawabata and Kawao, 2005)*

1.3.7. Role of PAR-2 in allergy

The role of PAR-2 in adaptive immune responses is less known than in innate inflammatory responses. Studies suggest a protective (D'Agostino *et al.*, 2007) as well as exacerbating (Schmidlin *et al.*, 2002; Ebeling *et al.*, 2005) effect of PAR-2 stimulation to allergic challenges. Stimulation of PAR-2 results in super oxide production and degranulation of eosinophils and mast cells.

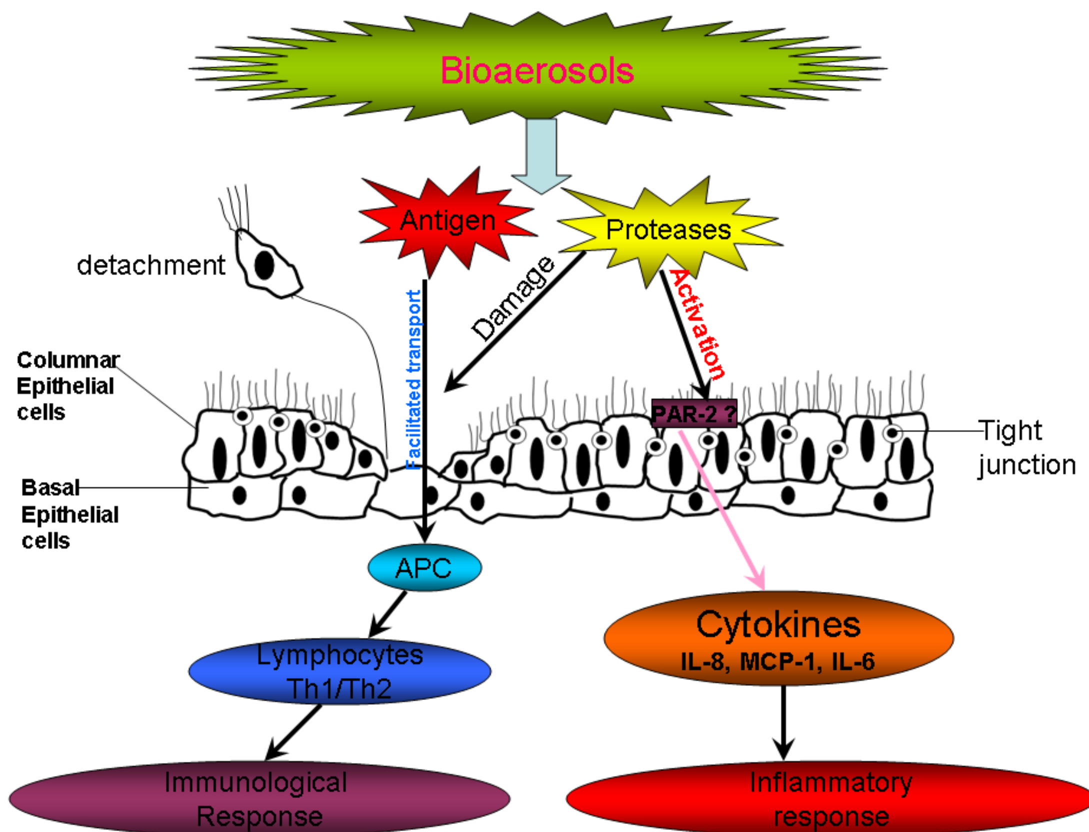

Fig.1.3. *Interaction of allergen proteases with epithelial cells. Cell damage and cytokine release are shown concomitantly with the proposed effects on the inflammatory response and facilitation of the immune response. APC, Antigen-presenting cells; MCP-1, monocyte chemotactic protein-1. Modified from (Kauffman et al., 2000)*

Epithelial cells have shown great importance in innate immunity (Bals and Hiemstra, 2004). They can also recognize microorganisms by surface receptors (Bals and Hiemstra, 2004). Crab antigen tropomyocin may be present in bioaerosols, produced at the production area in crab industry. Furthermore crab proteases like trypsin and chymotrypsin also play an important role in the innate inflammatory responses. Activation of PAR-2 induces cytokines (Tomee *et al.*, 1998) known to be important in inflammatory responses.

1.3.8. Desensitization of PAR-2

PAR-2 desensitization is initiated by the phosphorylation of *C*-terminal (Kumar *et al.*, 2007) that may lead to,

- receptor shutoff;
- further phosphorylation and receptor internalization;
- sequestration;
- proteolytic degradation of the receptor protein as well as the tethered ligand.

The mechanism of receptor internalization following phosphorylation is still unclear.

1.3.9. Disarming of PAR-2

Disarming is a phenomenon by which any receptor loose it's function due to "loss of arm". An example of disarming is, disarming of PAR-2 by cathepsin G and elastase.
During lung inflammation, airspaces are covered by neutrophiles that release elastase and cathepsin G (serine proteases). These proteases do not activate PAR-2 but rather disarm the receptor by cleaving it on disarming cleavage sites and prevents the activation by trypsin (Dulon *et al.*, 2003).

1.4. IL-8 as an indicator of inflammatory responses.

Lungs epithelium is the first tissue to contact with inhaled pathogens and shows the response by releasing inflammatory mediators and cytokines. Respiratory epithelial cells can synthesize and secrete many proinflammatory cytokines such as IL-8, IL-1, IL-6, GM-CSF (Cromwell *et al.*, 1992) which controls the cell behavior in physiological and pathological conditions.

During recent years serine proteases got more importance in the understanding of airway disease. Particularly the importance of tryptase (Krishna *et al.*, 2001) in the development of asthma. Serine proteases perform a role in the induction of cytokine hypersecreation in airways (Uehara *et al.*, 2003) and production of IL-8, as increased levels of IL-8 were found in asthma (Nocker *et al.*, 1996).

The A549 cells have shown higher levels of IL-8 after stimulation with serine proteases. The increased IL-8 levels have been observed previously in A549 cells via activation of protease activated receptors. Interleukin (IL)-8 is a marker of inflammatory response and in our studies IL-8 was used as an indicator of inflammatory response.

1.5. The seafood proteases.

1.5.1. Seafood

Seafood is processed by skilled workers in different workplaces. They include fishermen, workers in processing plants where seafood is heated, prepared, etc. Seafood can cause IgE-mediated allergic reactions when they are aerosolized (Malo and Cartier, 1993). Various organ or systemic manifestations can be seen in sensitized workers. During processing and cleaning procedures in seafood industry, organic particles are emitted into the air and workers display increased prevalence of airway symptoms (Douglas *et al.*, 1995; Bang *et al.*, 2005). Previous studies shows that in airway

epithelial cells, salmon trypsin is a potent activator of PAR-2 and PAR-2 activation is coupled to increased production of IL-8 (Larsen *et al.*, 2008).

1.5.2. Serine protease

Serine proteases are the catalytic enzymes in which one of the amino acids at the active site is serine. They are widely distributed in the human body as well as in fish and found in both single-cell and complex organisms.

Serine proteases can be classified into clans that share structural similarities (homology). The major clans found in humans, include the chymotrypsin, trypsin and elastase.In the history of evolution, serine proteases were basically digestive enzymes. In mammals, they evolved by gene duplication to perform functions in blood clotting, the immune system, and inflammation.

Serine proteases, such as mast cell tryptase, trypsin-like enzymes, and certain allergens are important in the pathogenesis of asthma. These proteases can activate PAR–2. PAR-2 and PAR-4 are activated primarily by trypsin-like proteases (Macfarlane *et al.*, 2001). Trypsin, tryptase, and coagulation factors VIIa and Xa are considered possible endogenous activators for PAR-2 in the airway (Ossovskaya and Bunnett, 2004).

1.5.3. Trypsin and trypsin-like proteases

Trypsin is a member of serine proteases used to digest food and is found in the digestive tract. It is also released by immune cells to help to destroy bacteria. Trypsin and trypsin like proteases can activate protease activated receptors in the airways and cause inflammatory reactions and broncoconstriction.

1.5.4. Mechanism of serine protease

Proteases or proteinases are enzymes that catalyze the hydrolysis of peptidic bonds. In serine proteases the catalysis is based on nucleophilic attack of the targeted peptidic bond. In many cases the nucleophilic property of serine proteases is improved by the presence of a histidine, held in a "proton acceptor state" by an aspartate. Aligned side chains of serine, histidine and aspartate build the catalytic triad common to most serine proteases.

1.6. Moricrase

Moricrase is generated from the hepatopancreas of kamachatka crab *paralithodes camachatica* (Rudenskaya *et al.*, 2000) and is a mixture of proteolytic enzymes including serine proteases, like trypsin. Moricrase containing ointments has shown activity in wound healing, treatment of burns and postoperative scars.

Table 1

Enzymes detected in moricrase *Modified from (Rudenskaya et al., 2000)*

Protease	M_r, kDa
Collagenolytic serine proteinase	28
Trypsin	29
Metalloproteinase	22
Carboxypeptidase	34
Aminopeptidase	220

1.6.1. Moricrase in our study

Moricrase is a rich source of serine protease and the previous research has shown evidence of effect of trypsin and trypsin like protease on the activation of protease-activated receptors in the airways. Workers of crab industries have shown airway symptoms including shortening of breath and asthma like symptoms. Moricrase contain enzymes which may have effect on the lungs, like trypsin and chymotrypsin. The workers at the crab processing area are expected to be exposed to airborne proteases (bioaerosols) produced in the crab processing procedure.

In our study we investigate the specific effect of moricrase on IL-8 levels in A549 cells and the activation of protease-activated receptor-2 by using specific siRNA for protease-activated receptor-2.

2. METHODS

2.1. General maintenance of cell culture.

2.1.1. Cell medium

Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (1:1), 1% Glutamine and 10% Fetal Bovine Serum (Gibco, NY, USA) was used to culture the cells. The components of the cell medium are:

<u>DMEM/F12:</u> contains a number of vitamins, cofactors, salts and carbohydrates necessary for cell growth support (Even *et al.*, 2006).

<u>L-Glutamine:</u> Glutamine provides support to the growth of cells requiring high energy demands and synthesizing large amounts of proteins and nucleic acids. It is an alternative energy source for rapidly dividing cells and cells that use glucose inefficiently.

<u>FBS:</u> Fetal Bovine Serum (FBS) was used in the medium for all the experiments
FBS is capable for supporting the growth of a variety of cell types. The availability and ease of storage of FBS (Barnes and Sato, 1980) together with its rich content of growth factors and low gamma-globulin content in comparison with other animal sera (Shah, 1999) have led to its adoption as the standard medium supplement.

<u>Phenol red:</u> Most living tissues prosper at a near-neutral pH; that is, a pH near to 7. The pH of blood ranges from 7.35 to 7.45, for instance. When cells are grown in tissue culture, the medium in which they grow is held close to this physiological pH. Waste products produced by dying cells or overgrowth of contaminants cause a change in pH, leading to a change in indicator (phenol red) color. For example, a culture of relatively

slowly-dividing mammalian cells can be quickly overgrown by bacterial contamination. This usually results in an acidification of the medium, turning it yellow. In addition, the waste products produced by the mammalian cells themselves will slowly decrease the pH, gradually turning the solution orange and then yellow.

2.1.2. Cell culture

The cells kept for stock of culture were not overcrowded in the flask and the medium was changed after each 48 hours in order to keep the cells healthy. While the cells used in the experiment were counted and approximately exact number of cells incubated for the experimental setup. In order to provide *in vivo* conditions the cells were kept in an incubator at 37° C with 5% CO_2 in air.

In order to perform good experiments with the cells, regular checking of cell culture was needed. Regular checking was to make sure that the cells were showing adherent behavior and growing accordingly, if not then a new ampoule was thawn. If there was any sign of infection then the flask was removed from the incubator so that infection should not spread in other flasks.

2.1.3. Cell counting

Living cells are distinguished from dead cells based upon the integrity of the cell membrane. Dead cells allow penetration of water soluble compounds (e.g. Trypan Blue 0.4%) that cannot cross intact plasma membrane and dead cells lose their cytosolic components by diffusion through the compromised membrane and turn blue. The cells were counted on Burker chamber which is divided in 9x16 fields.

In this procedure, membrane exclusionary dye, trypan blue 0.4%, was used to count and assess cell viability. Viability is given as a percentage of living cells in a cell culture.

$$\textbf{Viability} = \frac{\textbf{Number of living cells}}{\textbf{Number of living cells + dead cells}} \times 100$$

Procedure

1. A flask of growing cells were taken out from the stock of cells, 5 ml of cell dissociation solution were added in the flask and incubated in a CO_2 incubator (Maintained 5% CO_2 level at 37° C to simulate *in vivo* conditions) until all cells detached and started to float.
2. After 15 min if the cells were floating then the cells suspension were centrifuged for 5 min at 1100 rpm in spin centrifuge.
3. Supernatant was removed and pellet was dissolved in 1 ml of medium.
4. 20 µl of cell suspension and 180 µl of trypan blue 0.4% were mixed in a 1.5 ml micro tube.
5. 20 µl of the above mentioned solution was taken out, placed on Burker Chamber and the cells were counted.
6. Number of the cell were counted by using the following formula:

Total number of cells/ml = cells counted in 16 small squires x 10 x 10,000

(where 10 is dilution factor for trypan blue)

7. Living and dead cells were counted as shown in the Fig.2.1

Burker Chamber with cells diluted in Trypan blue 0.4%

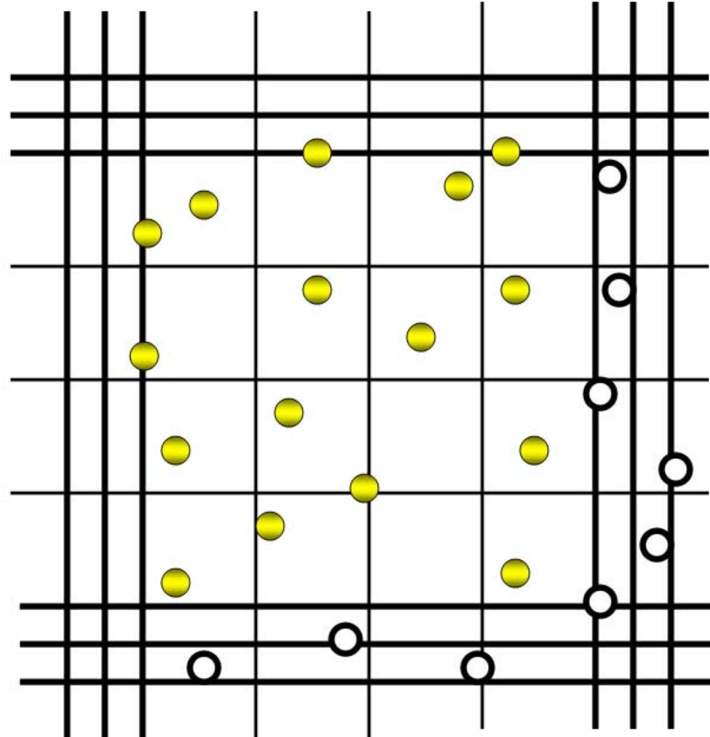

Fig.2.*1. Method of cell counting at Burker chamber. The cells in the square and which are touching the top and left border were counted* ***(O)****. The cells touching the right and lower border were not counted* ***(O)****.*

Modified from. http://www.fao.org/DOCREP/003/W3732E/w3732e0k.gif

2.1.4. Cell freezing

The freezing method was used to store the cells for longer time. Cells that were supposed to be frozen were free from infections with viability over 90%. For A549 cells 5-6 million cells were expected per T75 flask and freezing media was prepared accordingly.

Freezing Media	
DMEMF/12	**4.0 ml**
FBS	**0.5 ml**
DMSO	**0.5 ml**
Total volume 5.0 ml ice cold	

Procedure

1. A flask of growing cells were taken out from the stock of cells, 5 ml of cell dissociation solution were added in the flask and incubated in a CO_2 incubator (Maintained 5% CO_2 level at 37° C to simulate *in vivo* conditions) until all cells started to float.
2. After 15 min if the cells were floating then the cells suspension were centrifuged for 5 min at 1100 rpm in spin centrifuge.
3. Supernatant was removed and pellet was dissolved in 5 ml of freezing media.
4. 0.5 ml of cell suspension was aliquot in cryotubes (roughly 500 000 cells/tube) and frozen down at -70° C over night and transferred to the liquid nitrogen freezer for long term storage.

2.1.5. Cell thawing

A549 cells were thawn at room temperature, not any special equipment is required for thawing the cells. Medium (DMEM/F12) was taken to the incubator 15 min prior to start of the thawing procedure. The ampoule (cryotubes) was taken out from freezer and kept at room temperature in sterile area for 5 minutes to thaw. The cells were mixed with 1 ml of medium and centrifuged in a sterile centrifuge tube for 5 min at 1100 rpm.

Supernatant was removed and 1 ml of medium was added to dissolve the cell pellet. The cells were incubated in a T25 flask in 5 ml of medium (DMEM/F12, 10 % FBS, 1 % of Glutamine). The cells were expanded in T75 flask after getting 80-90% confluence.
The size of T-flask was dependent on the experiment design and number of cells needed. Seeding capacity for T25 flask is 1.5 x 10^6 and T75 flask is 5 x 10^6. The cell medium was changed after each 48 hours in order to get 80-90% confluency.

The cell culture was checked under microscope. If there were no signs of infection and cells were in healthy condition then the cells were used for the experiments. In order to get good results these observations were followed:

- Healthy growth usually leaves media slightly orange.
- Too yellow (milky) means bacterial growth.
- Too purple means low carbon dioxide, cells dead.
- In order to save time and to get good results the cells were checked daily. In the presence of infection (milky yellow colour or any kind of milky appearance) or if the cells are not growing then the cells were taken to autoclave and discarded and a new ampoule were thawn.

After thawing, the cells were treated as stock of cells which was used for further experiments.

2.2. Growth curve

A549 cells were purchased from American Type Culture Collection (ATCC; no CCL-185). A growth curve experiment was established to investigate viability and changes in behavior of A549 cells. Moreover to demonstrate the growth of A549 cells in our lab system and to obtain a population doubling time. A growth curve generally shows:

Lag phase, the time in which cells recover from subculture, attach and spread.
Log phase, in which the cell number increase exponentially.
Plateau phase, in which the cells become confluent and the growth rate slows or stop

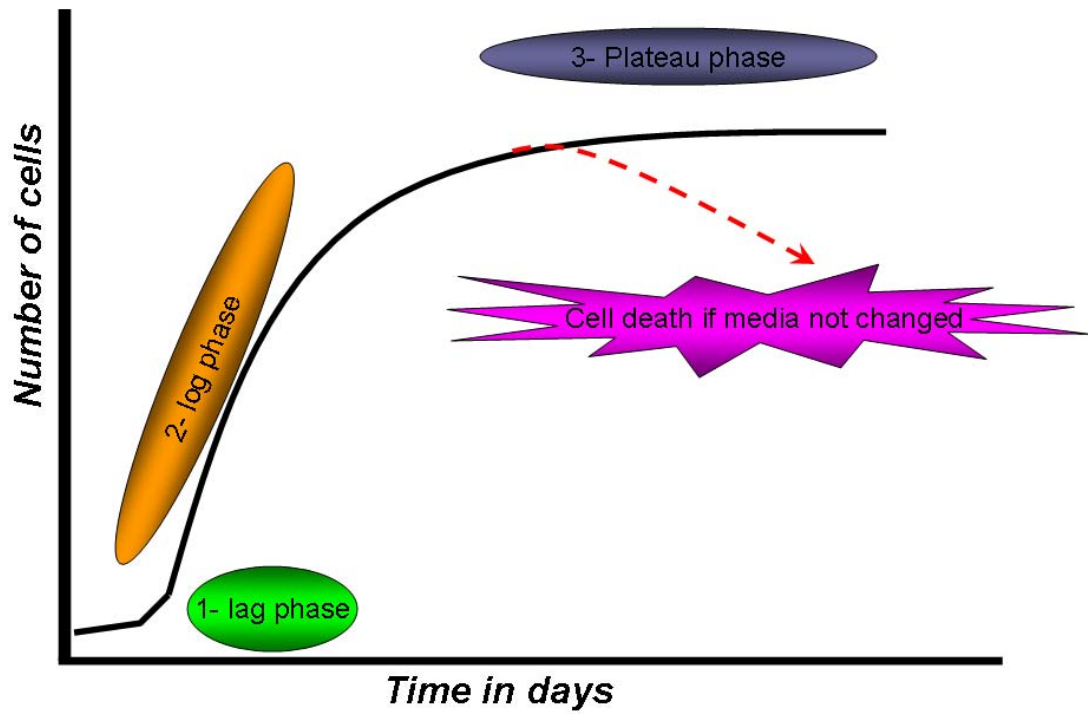

Fig.2.2

Graph showing the logarithmic cell growth and the plateau stage where the growth slows greatly and if the media was not changed, cells start dying.

Modified from

http://www.bme.gatech.edu/vcl/Tissue_Engineering/Background/5_cell_seeding.htm

2.2.1. Doubling time

The doubling time is a time require for cells to double in numbers. Estimated doubling time for A549 cell line was 22 hours according to supplier (ATCC). Estimated viability was 97%. The population doubling time of A549 cells was calculated by using the formula:

Ln2/b= T2

Where **ln2** is natural log 2. **T2** is doubling time and **b** is slope of increasing number of cells in log phase.

Procedure

1. A flask of growing cells were taken out from the stock of cells, 5 ml of cell dissociation solution were added in the flask and incubated in a CO_2 incubator until all cells started to float.
2. After 15 min, if the cells were floating, then the cells suspension were centrifuged for 5 min at 1100 rpm in a spin centrifuge.
3. Supernatant was removed and pellet was dissolved in 1 ml of medium.
4. 180 µl of trypan blue 0.4% was mixed with 20 µl of cell suspension in a 1.5 ml micro tube.
5. For 12 days 150 000 x 12 = 1 800 000 cells were sufficient to setup the experiment.
6. 5 ml of medium and 150 000 cells were added in each T25 flask and incubated in CO_2 incubator at 37° C.
7. For growth curve experiments, the cells were observed for 12 days without changing the medium, the dead and live cells were counted after each 24 hours.
8. The cell population doubling time in the log phase was calculated by the above given formula.

2.3. Enzyme activity

Enzyme activity is a measure of the quantity of active enzyme. A stock solution of moricrase 5 mg/ml were prepared, by dissolving a solid freeze dried moricrase in dH_2O and was examined under spectrophotometer for the calculation of enzymatic activity.

2.3.1. Trypsin-like activity

Trypsin is a serine endopeptidase that is formed from trypsinogen in the pancreas. It is converted into it's active form by enteropeptidase in the small intestine. It catalyzes hydrolysis of the carboxyl group of either arginine or lysine.

Substrate for trypsin: Nα-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride (DL-BAPNA, Product number B4875) is a chromogenic substrate for proteolytic enzymes such as trypsin, amidase, and balterobin.

Preparation of stock solutions:

- Stock solution of trypsin was made at the concentration of 1 mg/ml. The solution were filtered through sterile filter and frozen down at -20° C for further use.
- Substrate for trypsin was prepared as stock of 100 millimolar DL-BAPNA.70.16 mg of DL-BAPNA were mixed with 1.6 ml of DMSO and stored at -20° C.

Substrate buffer:

25 millimolar Tris/HCl, 10 millimolar $CaCl_2$, 2 % (v/v) DMSO, pH 8.1

1.97g Tris/HCl and 0.687g $CaCl_2$ were dissolved in 300 ml of dH_2O. 10 g of DMSO were added to the solution and the pH was adjusted to 8.1 by using NaOH final volume were adjusted to 500 ml and stored at room temperature.

2.3.2. Chymotrypsin-like activity

Chymotrypsin is a serine endopeptidase secreted by the pancreas as its zymogen, (chymotrypsinogen) and carried in the pancreatic juice to the duodenum where it is activated by Trypsin. It selectively cleaves aromatic amino acids on the carboxyl side.

Substrate for chymotrypsin: N-Succinyl-Ala-Ala-Pro-Phe-P-Nitroanilide (N-suc-AAPF-pNA, product number S7388)

Substrate buffer:

0.1M Tris/ HCl, 0.005% Triton X-100, p H 8-8.5

15.6 g of Tris/ HCl was dissolved in 800 ml of dH_2O. 5.3 g of triton x 100 was transferred to the Tris/HCl solution; pH was adjusted between 8 to 8.5. Final volume was adjusted to 1000 ml and stored at room temperature

Procedure

1. 5x dilution series were prepared from enzyme stock; dilutions were made in substrate buffer
2. 96-well plate was used and various samples were investigated, 10 µl/ well, and 3 wells for each dilution.
3. Substrate solution was added in appropriate wells and optical density was measured at 405 nm for 5 minutes (for chymotrypsin-like activity) and 10 minutes (for trypsin-like activity).
4. The curve of the measurement has to be below 60°∟ in order to have an acceptable limit.
5. An average of 3 parallels was taken and the enzyme activity was calculated in the sample.

Units and calculations for trypsin like activity and chymotrypsin like activity:

1 unit = da/dt x 1/(ε x 1) x 10^6 x V_{final}

1 = 0.709 cm (light path)

V_{final} = 0.000251

ε = 8800 M^{-1} cm^{-1}

dA/dt = mOD/min x 10^{-3}

1 U = 4.0 x 10^{-5} x mOD/min

1U/ml = 4.0 x 10^{-5} x 1000/10 = 4.0 x 10^{-3} mOD/min

Enzyme activity for trypsin like activity and chymotrypsin like activity = Average of test sample for each solution × 4.0 × 10^{-3}

2.4. Dose Response

The science of toxicology as well as pharmacology is based on the principle that there is a relationship between a reaction (the response) and the amount of substance received (the dose). An important assumption in this relationship is that there is almost always a dose below where no response occurs. A second assumption is that once a maximum response is reached any further increases in the dose will not result in any increased effect (Anonymous, 1993).

Dose response study is a method to determine the relationship between dose of stressor and a specific biological response. In this case we use it to evaluate the quantitative relationship between dose and toxicological response.

Procedure

1. A549 cell culture was expanded until the required number of cells to setup the experiment.
2. The experiments were performed in 24 well plates and the seeding density was 150 000 cells/well and each well contained 350 µl of medium.
3. In order to get good results and to avoid any kind of mistakes two parallel experiments were performed together.
4. The medium was changed after 24 hours.
5. Wells were washed 3 times with serum free medium DMEM/F12 (1:1) on the second day, to remove the traces of FBS. After washing three times the cells were incubated with DMEM/F12 over night.
6. On the third day, the cells were stimulated with moricrase**.** The following concentrations of moricrase were used:

0.0166 µg/ml

0.03325 µg/ml

0.0665 µg/ml

0.137 µg/ml

0.266 µg/ml

0.532 µg/ml

7. Tumor necrosis factor (TNF) alpha was used as positive control.

- TNF alpha stock solution was 100 ng/µl. 1:100 dilutions were required so 1 µl of TNF alpha stock solution were added in 99 µl dH2O.
- 10µl of the above mentioned solution were added in 1000 µl of serum free DMEM/F12 and this solution were used as a positive control.

8. DMEM/F12(without serum) was used as negative control.
9. Volume/well: 350 µl
10. Stimulation solutions were kept at 37° C for 15 minutes before adding them to the cells.
11. The medium was removed from the wells and stimulation solution was added.
12. The wells were incubated for 6 hours.
13. After 6 hours the morphology of the cells were evaluated under microscope and supernatant was frozen down at -20° C for ELISA.
14. The cells were taken to autoclave and discarded.

2.5. Time response

A study of biological response from a specific biological material at specific time is known as time response studies. In the time response studies our best stimulation concentration of moricrase (0.03325 µg/ml was used. (Concentration was obtained from dose response experiment.)

Procedure

1. **1.** Steps 1-10 (except step 6) were similar to dose response studies.
2. **2.** The medium was removed and 0.03325 μg/ml concentration of moricrase was added in all wells.
3. **3.** The wells were incubated for 6, 12, 18, 24 and 48 hours.
4. **4.** After each time period, morphology of the cells was evaluated under microscope and supernatant was frozen down at -20° C for performing ELISA.

2.6. ELISA

The amount of secreted IL-8 in the supernatant was determined by using an ELISA (enzyme-linked immunosorbent assay) kit from BD Biosciences, NJ, USA, according to the manufacturer's protocol. The absorbance was measured by using an iEMS Multiscan EX (Thermo Lab systems). IL-8 production was expressed as pg/ml.

The IL-8 Kit is a solid phase sandwich ELISA. A monoclonal antibody specific for IL-8 is coated onto the wells of the microtiter strips. Samples, including standards of known IL-8 concentrations, control specimens and unknowns were pipetted into these wells.

During the first incubation, the IL-8 antigen and a biotinylated polyclonal antibody specific for IL-8 were simultaneously incubated. After washing, the enzyme (streptavidin-peroxydase) was added. After incubation and washing to remove the entire unbound enzyme, a substrate solution which is acting on the bound enzyme was added to induce a coloured reaction product. The intensity of this coloured product was directly proportional to the concentration of IL-8 present in the samples.

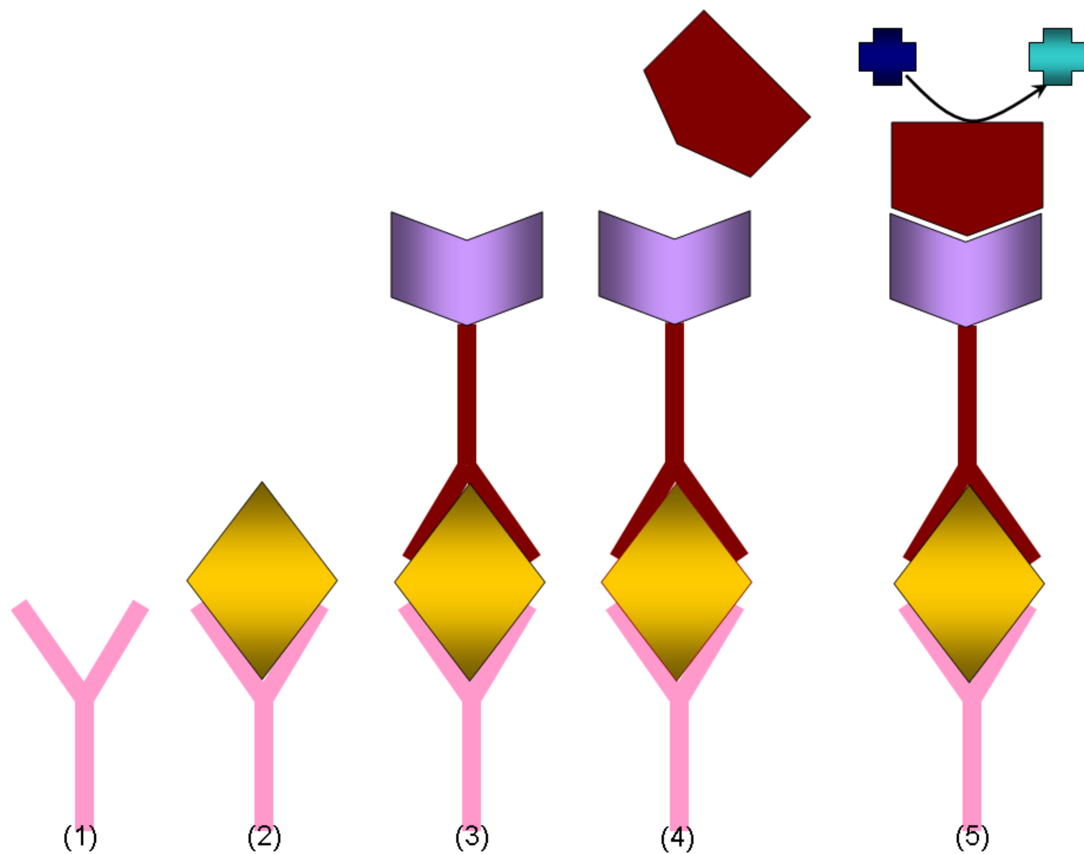

Fig.2.3

(1) ELISA plate is coated with a capture antibody; (2) Sample is added, and the respective antigen present binds to capture antibody; (3) Biotin-conjugated secondary detection antibody is added, and binds to the antigen captured by the first antibody; (4) Streptavidin-HRP is added and binds to the biotin conjugated detection antibody; (5) Coloured product is formed in proportion to the amount of antigen present in the sample; The reaction is terminated by addition of acid and absorbance is measured at 450 and 540 nm. Modified from. http://www.bendermedsystems.com/elisa--22

Table 2

Solutions used in ELISA kit and their contents

Modified from supplier's protocol

ELISA Solutions	**Contents**
Detection antibody	30 ml of biotinylated anti-human IL-8 monoclonal antibody containing FBS and ProClin™-150 as preservative
Standard	lyophilized recombinant human IL-8
Enzyme concentrate (250×)	150 µl of 250× concentrated Streptavidin-horseradish peroxidase conjugate with BSA and ProClin™-300 as preservative
Standard/Sample diluent	30 ml of animal serum with 0.09% sodium azide as preservative
ELISA diluent	12 ml of a buffered protein base with 0.09% sodium azide as preservative
Wash concentrate (20×)	100 ml of 20× concentrated detergent solution with ProClin™-150 as preservative
TMB substrate reagent	30 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution
Stop solution	13 ml of 1M phosphoric acid
Working detector	For a full 96-well plate, 48 µl of Enzyme Concentrate into 12 ml of Detection Antibody

Solution preparation

All reagents and samples were brought to room temperature (18 – 25° C) 30 minutes prior to use. All standards and samples were run in duplicate. A standard curve was prepared in each assay run.

Standards

- 1 vial lyophilized Standard with required volume (noted on vial label) of Standard/Sample Diluent were reconstituted to prepare a 200 pg/ml stock standard.
- In order to make dilutions, 300 µl Standard/Sample Diluent was added to 6 tubes. Labeled as 100, 50, 25, 12.5, 6.3, and 3.1 pg/ml.
- Serial dilutions method was used by adding 300 µl of each standard to the next tube and vortexing between each transfer. The undiluted standard was used as the high standard (200 pg/ml). The Standard/Sample Diluent was used as the zero standard (0 pg/ml).

Samples

- 1: 20 dilutions of all samples (except TNF alpha) were prepared. For TNF alpha, 1:80 dilutions were prepared. For dilutions “standard/sample Diluent” (provided in the ELISA kit) was used.

Wash Buffer

- 100 ml of wash concentrate were mixed with 1900 ml of distilled water and 2 litter of wash buffer was prepared.

Procedure

1. The required quantity of test strips/wells were removed and placed in well holder.
2. 50 µl of "ELISA Diluent" were pipette into each well.
3. 100 µl of each standard and sample was pipette into appropriate wells, the wells were covered by plate sealers and incubated for 2 hours at room temperature.
4. Required volume of "Working Detector" was prepared (15 minutes before use) according to the instruction manual.
5. The wells contents were aspirated and washed with 300 µl/well "wash buffer" for a total of 5 times. Absorbent paper was used after the last wash to remove any residual buffer.
6. 100 µl of prepared "Working Detector" were added to each well; the wells were covered by plate sealer and incubated for next 1 hour at room temperature.
7. The wells were washed in the same procedure as above but total 7 times.
8. 100 µl of "TMB One-Step Substrate Reagent" (provided by the supplier) were added to each well. The plate were incubated (without Plate Sealer) for 30 minutes at room temperature in the dark.
9. 50 µl of "Stop Solution" (provided by the supplier) were added to each well.
10. Absorbance was measured at 450 and 540 nm within 30 minutes of stopping reaction.
11. Absorbance reading at 540 nm was subtracted from reading of 450 nm. This subtraction is for correction of optical imperfections in the plate.

Calculation

1. The mean absorbance for each set of duplicate standards, controls and samples were calculated. The mean zero standard absorbance (i.e. absorbance of diluent) was subtracted from each value.
2. The standard curve on log-log graph paper was plotted, (as shown in Fig. 2.4) with IL-8 concentration on the x-axis and absorbance on the y-axis. The best fit straight line was drawn through the standard points.
3. To determine the IL-8 concentration of the samples, the samples's "mean absorbance value" was found out on the y-axis and a horizontal line was drawn to the standard curve. At the point of intersection, a vertical line was drawn to the x-axis and red the IL-8 concentration. If samples were diluted, the interpolated IL-8 concentration was multiplied by the dilution factor.

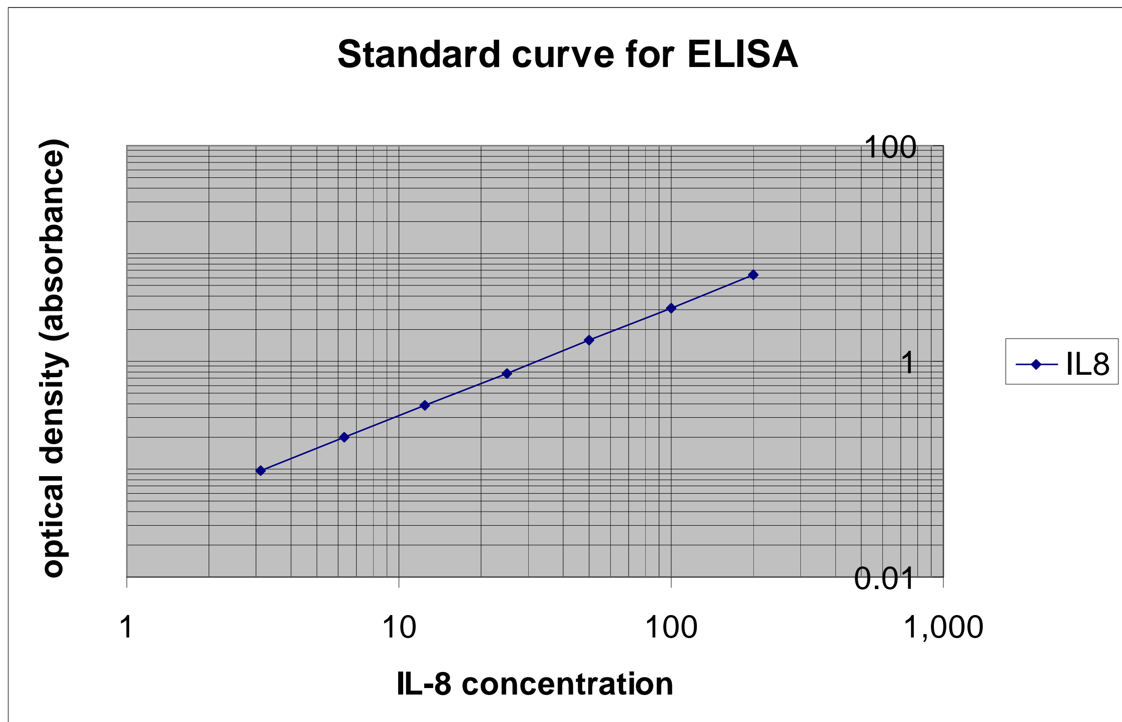

Fig. 2.4. *Standard curve for the calculation of ELISA results. The standard curve was prepared by using the optical density of standard dilutions. Standard curve was plotted for every experiment according to the absorbance data of standard solution dilutions.*

2.7. Small interfering RNA (siRNA)

Small interfering RNA. A mix of three pre-designed siRNAs (Ambion, USA, catalogue number 16704, ID nr 1960, 1876, and 1783) for the PAR-2 gene was used. The intention was gene silencing or suppression of PAR-2 by using siRNA to study the reduction in the production of IL-8 after stimulation.

RNA interference (RNAi) represents a mechanism invented by nature to protect the genome. The molecular mechanism of gene silencing provides with small interfering RNAs (siRNAs) which allows targeting any gene with high specificity and efficiency.

2.7.1. Mechanism of siRNA silencing

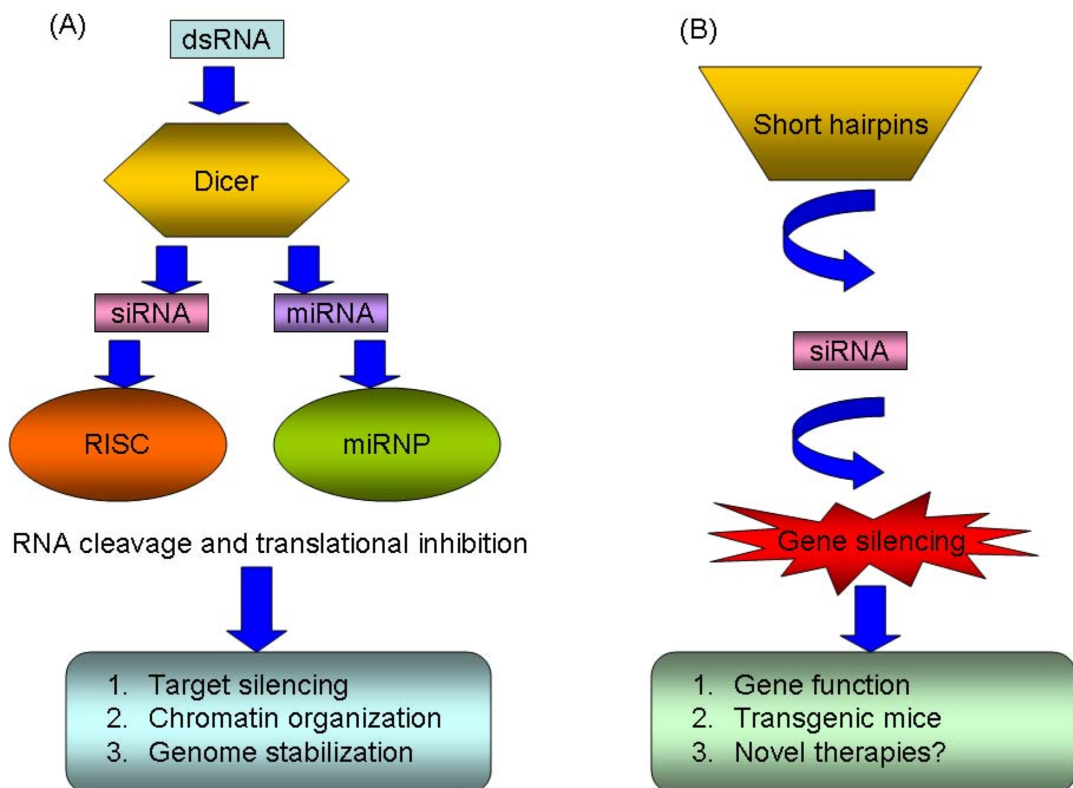

Fig.2.5 *siRNAs as mediator of RNAi and as a tool for gene function analysis. (A) dsRNA from various sources is cleaved by the Dicer "multi protein complex" to generate short duplexes (siRNAs) or single stranded short RNAs (miRNAs). "Multi protein complexes" then are targeted to complementary RNA species to mediate gene silencing. siRNAs become part of an "RNA-inducing silencing complex" which mediates gene silencing by target RNA cleavage. A related miRNA protein complex is able to silence genes by either RNA cleavage or translational inhibition. Both complexes differ by associated proteins. (B) This mechanism can be used to analyze gene function in vitro and in vivo. Short hairpins generated from vector systems in cells are converted to siRNAs (which can also be applied exogenously) thus mediating gene silencing.* Modified from (Schutze, 2004)

A549 cells (150 000 cells/well) were seeded in 24-well plates and were transfected by using Lipofectamine 2000 (Invitrogen). Transfection was performed according to the manufacturer's protocol.

Transfection of animal cells typically involves opening transient pores or "holes" in the cell membrane, to allow the uptake of siRNA. [http://en.wikipedia.org/wiki/Transfection] Lipofectamine 2000 is a cationic liposome formulation that functions by complexing with nucleic acid molecules, allowing them to overcome the electrostatic repulsion of the cell membrane and to be taken up by the cell (Dalby *et al.*, 2004).

Procedure

In order to stimulate A549 cells with moricrase, 5 mg/ml solution of moricrase was used as stock. The cells were also stimulated with PAR-2 peptide (4 mg/ml). For silencing of receptor; siRNA PAR-2 was used. The Silencer negative control nr 1 (Ambion, catalogue number 4611) was used as a negative control siRNA

0.03325 µg/ml concentration of moricrase was used for stimulation of A549 cells.
The receptor of PAR-2 was blocked by using siRNA PAR-2.

The effect of transfection was evaluated by using siRNA negative control.

Lipofectamin solutions.

- 1500 µl of OptiMem (reduced serum medium) was mixed with 30 µl of Lipofectamin (Lipofectamin 1 µl/well).
- Incubated for 5 minutes at room temperature.
- SiRNA-PAR-2: siRNA nr, 1960, 1876, 1783, 50 µl x 12 wells = 600 µl + 600 µl Lipofectamin solution were mixed.
- siRNA negative control, 50 µl x 12 wells = 600 µl + 600 µl Lipofectamin solution were mixed together.
- These Lipofectamin solutions were used as transfection solutions and were made 20 minutes prior to perform transfection.

1. On the first day 24 well plates were prepared. 150 000 cells/well were seeded out with DMEM/F12, 10% FBS, 1% glutamine. Three parallels of each sample were used.
2. The old medium was removed and new medium was added on the second day.
3. On the third day the old medium was removed and the cells were washed 3x with serum free medium.
4. The above mentioned transfection solution (Lipofectamin) was incubated for 20 min at room temperature.
5. 400 µl of OptiMem and 100 µl of transfection solution were added per well.
6. Transfection solution was added drop by drop while gentle shake of the tray in order to get proper mixing.
7. For completing the transfection procedure, 24 well plates again incubated for 4 hours. In 5% CO_2 incubator at 37° C.
8. After 4 hours, transfection solution was removed.
9. Wells were washed and rinsed with 500 µl of serum free medium DMEM/F12. New serum free medium was added and incubated over night.

10. On the forth day old medium was removed and the wells were washed 3x with serum free medium.
11. Stimulation of transfected and non-transfected A549 cells with moricrase, positive control (PAR-2 peptide (SLIGRL(NH2), and negative control DMEM/F12 were taken on the same day (4th day)
12. After 6 hours incubation the supernatant was collected and frozen at -20° C until ELISA.

3. Results

3.1. Characterization of A549 cell growth in our culture system.

In order to determine the behavior of A549 cells, a growth curve experiment was established. The cell line have shown lag phase, log phase, plateau phase and cell death phase. Fig 3.1 (a, b, c) shows that in the first three days (lag phase) the cells were sticking to the plastic and adjusting according to the environmental condition, while from 3 to 7 days it was a log phase where the cells reproduced exponentially. Day 7 to day 8 gives a picture of plateau phase and cells started to degrade after day 8.

The number of dead cells in a culture was increasing day by day, starting from approximately 1 and ends almost at 2 million. Maximum number of cells (dead and live in a medium) was more than 5 million in each experiment. The calculated mean population doubling time for A549 cells in three different experiments was 30.33 ± 2 (Mean ± Standard Deviation) hours. The growth curve experiment was started with approximately 99% viability which was reduced to half at day 12.

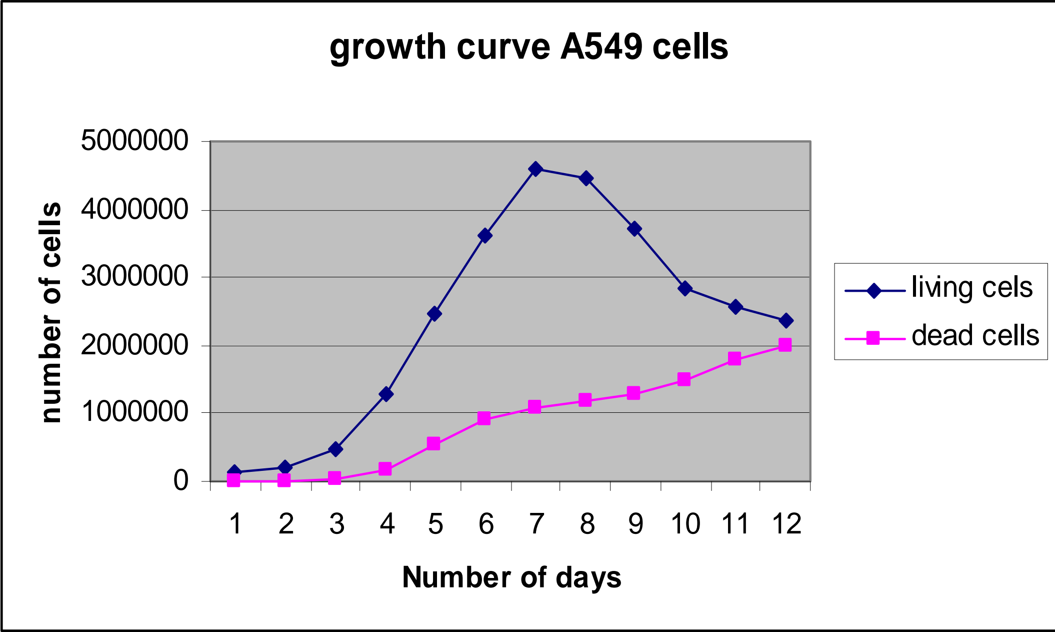

Fig.3.1 (a)

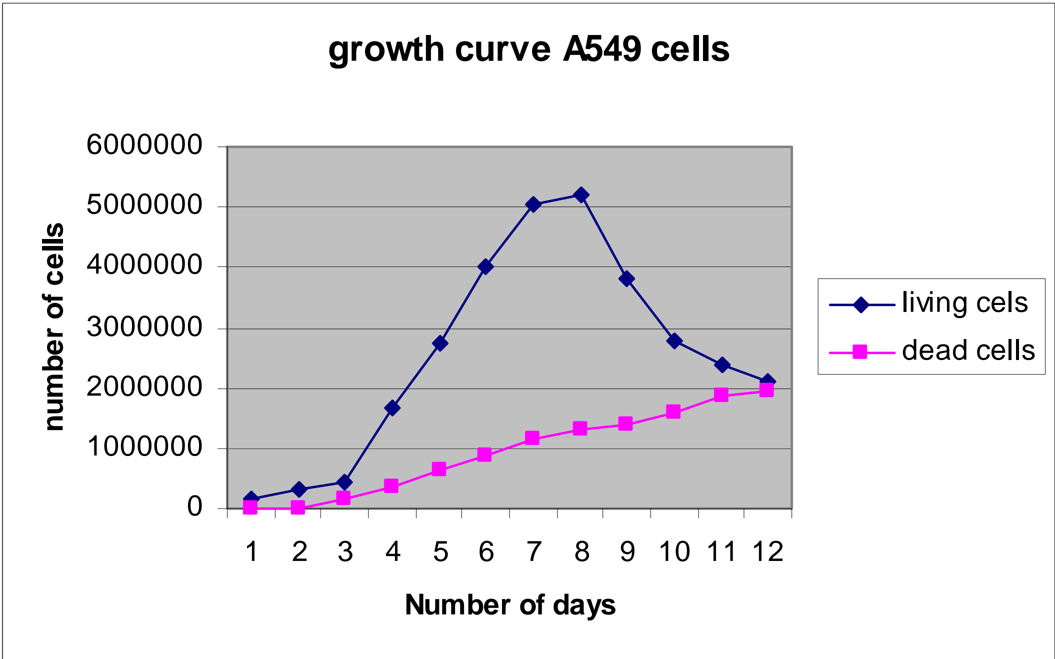

Fig.3.1 (b)

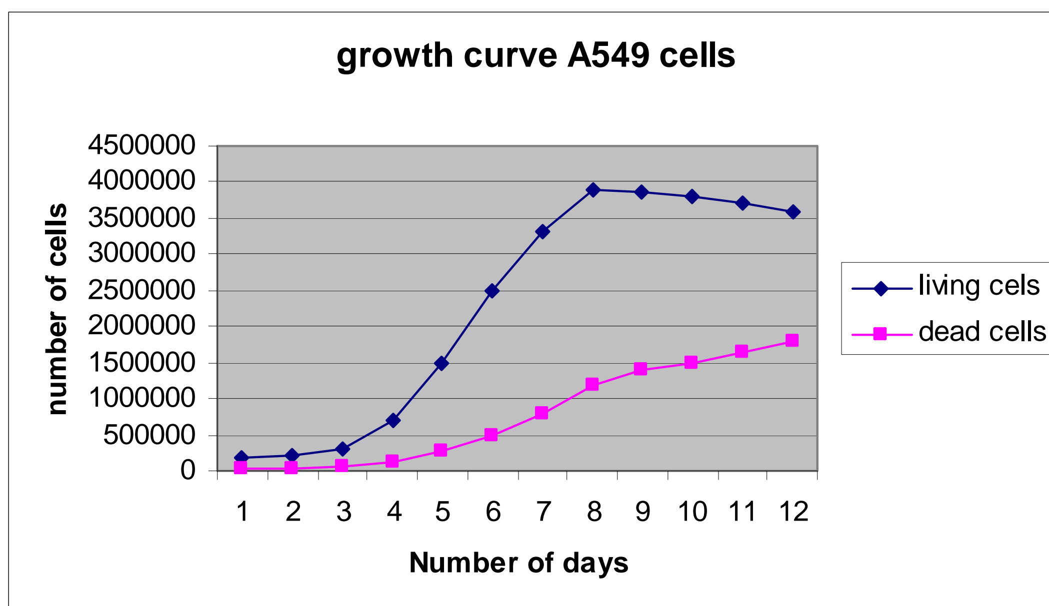

Fig.3.1(c)

Fig.3.1 (a, b, c) *Growth curve of A549 cell line. A549 cells were seeded out in 12 T25 flasks in DMEM/F12. Approximately* 1.5×10^5 *cells were seeded per flask. Every day one flask has been taken for counting the cells. The cells were counted by using Burker's chamber.*

In the growth curves blue lines shows the growth of living cells; while the red line indicates the number of dead cells. As the days passed the numbers of living cells were increased up to the maximum confluence. $4.5\times10^6\pm 6.5\times10^5$ (n=3) living cells were present at the day 7.66±0.57 (n=3). Approximately 1 million dead cells were found (in all 3 experiments) on the same day. After day 7 the number of living cells reduced in the medium while the numbers of dead cells were increased to approximately 2 million on the 12th day.

Table 3

experiment	Start cell concentration (live)	Maximum cell concentration (live)	Day of maximum cell concentration (live)	Doubling time (hours)
A	150 000	4 600 000	7	29.9
B	150 000	5 200 000	8	28.5
C	175 000	3 900 000	8	32.6
Mean ± S.D	$1.6\times10^{5}\pm1.4\times10^{4}$	$4.6\times10^{6}\pm6.5\times10^{5}$	7.66±0.57	30.33±2

Table shows a complete overview of all three growth curve experiments. The table shows only the living cells in the culture the dead cells were counted but not shown in the Table.

3.2. Activity of PAR-2 stimulating serine proteases in Moricrase.

To determine the presence and activity of serine proteases (known PAR-2 stimulators) in the moricrase, the enzymatic activity of moricrase regarding serine proteases were examined.

Table 4

Enzyme	**Substrate buffer**	**Specific activity of moricrase (U/ml)**
Chymotrypsin-like activity	N-Succinyl-Ala-Ala-Pro-Phe-P-Nitroanilide	0.0512
Trypsin like-activity	Na-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride	0.0621

3.3. Moricrase stimulation of IL-8 production in A549 cells

In order to determine the production of IL-8 after stimulation of A549 cells with moricrase, different studies were taken.

3.3.1 Concentration – response studies

To explore the concentration response relationship, different concentrations of moricrase was used. The cells were stimulated with 0.0166, 0.03325, and 0.0665 µg/ml, of moricrase solution. As shown in the Fig. 3.2 (a) The Fig shows that there is a gradual increase in the production of IL-8 as the concentration of moricrase is increased. The basal (DMEM/F12) production of IL-8 was stable at 200 pg/ml. At 0.0166 µg/ml there is only 30% increase in IL-8 production above basal level while at 0.03325 µg/ml it increased to 90 % and finally at 0.0665 µg/ml of moricrase the production of IL-8 was more than 1.5 times above basal level. The maximum IL-8 production in this experiment was 520 pg/ml at 0.0665 µg/ml while the basal IL-8 level was at 200 pg/ml.

It is also shown in the figure that the production of IL-8 is in increasing mode at 0.0665 µg/ml which means there may be more increase in IL-8 production with increasing concentrations of moricrase. In order to see the maximum response, three higher concentrations were used in the next experiment (b) and (c).
To determine the maximum response of a specific concentration 0.137 µg/ml, 0.266 µg/ml and 0.532 µg/ml were also used in the experiment as shown in Fig. 3.2 (b). The result shows that the concentration 0.0332 µg/ml has produced the maximum IL-8 (1400 pg/ml) in 6 hours incubation. The basal level (DMEM/F12) was stable at 500 pg/ml. There was almost two times increase in IL-8 production over basal level at

0.03325 μg/ml. The production of IL-8 was decreased (after 0.03325 μg/ml) with increasing concentrations of moricrase.

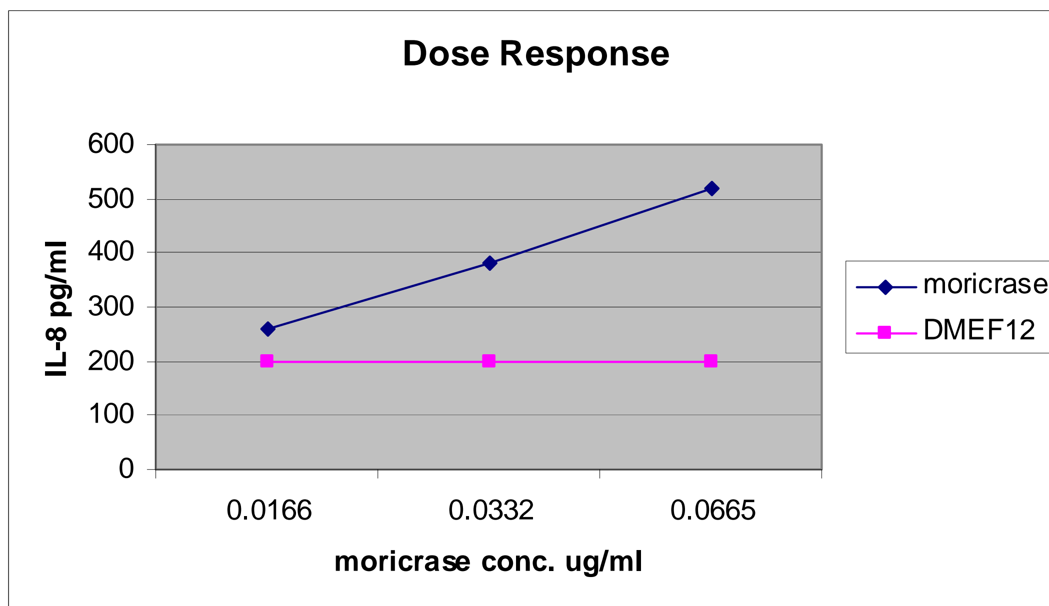

Fig. 3.2 (a)

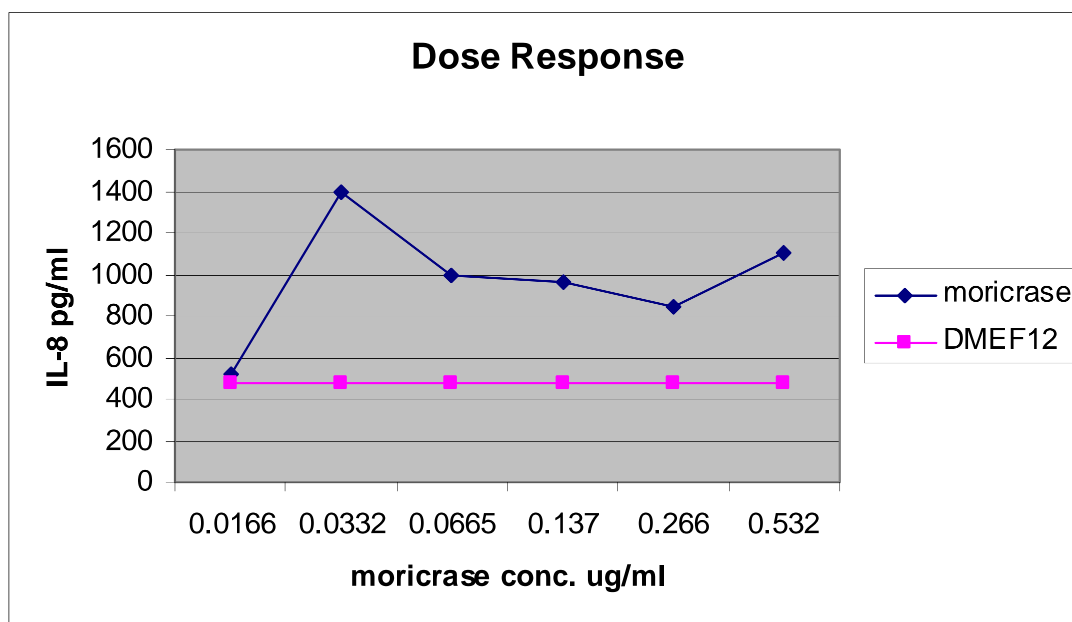

Fig. 3.2 (b)

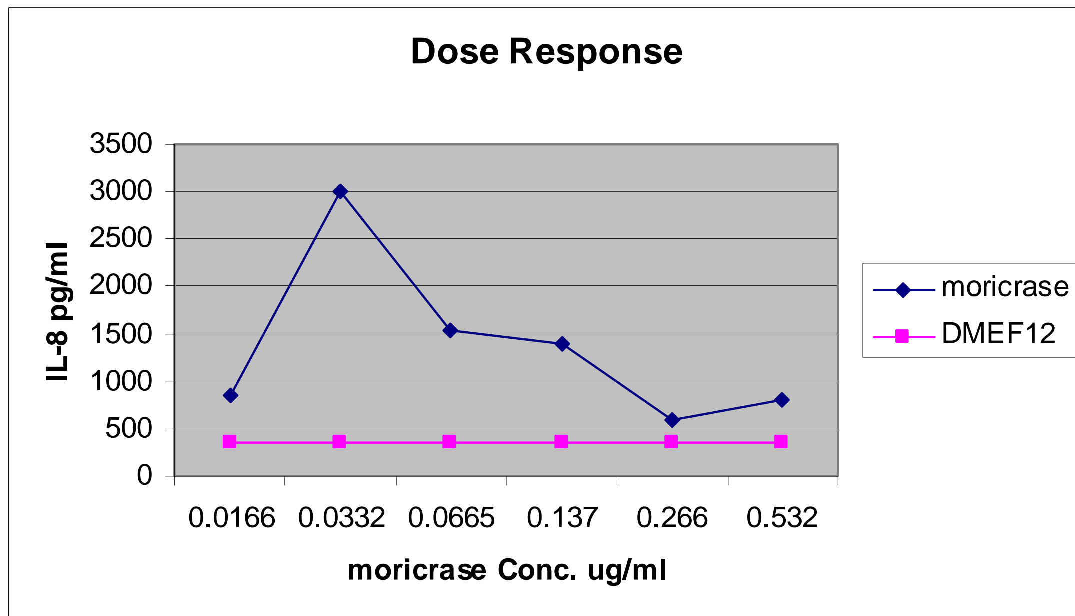

Fig. 3.2 (c)

Fig. 3.2. (a, b, c) *Effect of different concentrations of moricrase on the secretion of IL-8 from A549 cells. A549 cells (1.5 × 10^5 cells/well) were seeded out in DMEM/F12 in 24 well plates. 24 hours later the medium was changed. After next 24 hours the cells were incubated with the indicated concentrations of moricrase. The supernatant was harvested 6 hours later and was analyzed for the presence of IL-8 by ELISA.*

In order to confirm the maximum stimulation concentration, the same dose response experiment was setup and it was confirmed that the moricrase stimulates A549 cells and give the maximum production of IL-8 at the concentration of 0.03325 µg/ml as shown in the Fig.3.2 (c). The maximum production of IL-8 was 3000 pg/ml while the basal level was at 360 pg/ml. There was almost 1.5 times more increase in the IL-8 level compared to the basal level at the concentration of 0.0166 µg/ml while the production of IL-8 compared to 0.03325 µg/ml was decreased in the next higher concentrations (0.0665, 0.137, 0.266, and 0.532 µg/ml). The maximum production of IL-8 was more than 7 times above basal level at the concentration of 0.03325 µg/ml.

Table 5

Mean values and standard deviations of dose response

	Experiment (a)	Experiment (b)	Experiment (c)	Mean ± SD
Basal Level of IL-8 (pg/ml)	200	500	360	353 ± 150.1
Maximum level of IL-8 (percentage above basal level)	160	191.6	733	361 ± 322.2
Concentration of moricrase at maximum level (µg/ml)	0.0665	0.03325	0.03325	ND

ND = Not Determined

3.3.1.1. Morphological studies of dose response.

To determine the cell behavior after stimulation with different concentrations, morphology of the cells were studied in the dose response experiment. We found that for 6 hours stimulation of A549 cells with moricrase the cell adherence affected over the concentration of 0.0665 µg/ml. We examine under the micro scope (as shown in Table 6) that from 0.0166 µg/ml to 0.0665 µg/ml the cells were adherent but they have started detachment at 0.137 µg/ml which increased as the concentration increased and finally at the concentration of 0.532 µg/ml after 6 hours stimulation the cells were almost floating in the medium.

Table 6

Concentration of crab cocktail (µg/ml)	Morphology under microscope	Time of incubation in hours
0.0166	Cells were adherent	6
0.03325	Cells were adherent	6
0.0665	10% cells were rounded	6
0.137	20-25% cells were floating in the medium (detachment)	6
0.266	More than 50% cells were floating in the medium	6
0.532	Approximately 90% cells were floating in the medium	6

3.3.2. Time-response studies.

To explore the time-response relationship in IL-8 stimulation by moricrase in A549 cells, experiments of time response was established. A549 cells were stimulated with the best stimulation concentration 0.0332 µg/ml (calculated by dose response) for 6, 12, 18, 24 and 48 hours.

The results depicted in Fig.3.3 shows that production of IL-8 in A549 cells after stimulation with 0.03325 µg/ml of moricrase was increasing as the incubation time increased and have shown the maximum production of IL-8 after 24 hours and finally reduced after 48 hours.

In the first six hours the production of IL-8 was 55.5% above the basal level. After 18 hours the percentage production of IL-8 reached to 70% above basal level. Finally the maximum stimulation was shown at 24 hours incubation which was 90% above basal level while the cells were detached while after 48 hours the cells have shown detachment.

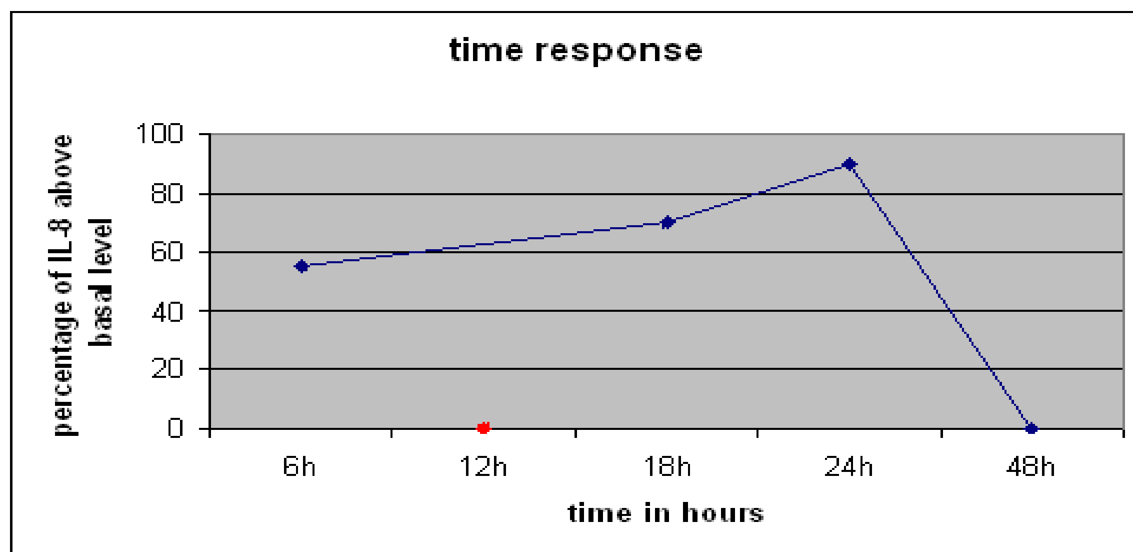

Fig.3.3 *Effect of moricrase stimulation on the secretion of IL-8 from A549 cells in different stimulation times. A549 cells (1.5 × 10^5 cells/well) were seeded out in DMEM/F12, FBS and Glutamine in 24 well plates, the cells were stimulated with the concentration of 0.03325 µg/ml and was incubated for 6, 12, 18, 24 and 48 hours. In order to give the similar conditions to all samples, six different plates were used. After each time period the sample's supernatant was collected and was analyzed for the presence of IL-8 by ELISA.*

(■) The drop at 12 hours was probably due to artifact, since this drop was not seen in previous or later experiments. This experiment is shown individually and other parallel experiments have been omitted due to some technical and contamination problems. The x-axis is presented as percentage above basal level as the basal levels have also shown increase in IL-8 stimulation.

3.3.2.1. Morphological studies of time response.

In order to determine the cell behavior after stimulation with 0.03325 µg/ml of moricrase at different times, morphological studies were carried out. In our time response studies the cells have shown an adherent behavior in the first 18 hours (as

shown in Table 7) and have shown less adherence in the next 6 hours (24 hours) and finally we have found approximately 80% cells floating in the medium after 48 hours incubation.

Table 7

Time of incubation in hours	Morphology under microscope	Concentration of crab cocktail in µg/ml
6	The cells were adherent	0.03325
12	The cells were adherent but approximately 2% of cells were rounded	0.03325
18	More than 20% cells were rounded	0.03325
24	Approximately 50% cells were round and 5% were floating	0.03325
48	Almost 80% cells were floating in the medium	0.03325

3.4. The involvement of protease activated receptor-2 on production of IL-8

3.4.1. PAR-2 peptide induces the secretion of IL-8 in A549 cells.

To determine whether PAR-2 activation could enhance the secretion of IL-8 in human airway epithelial cells, A549 cells were stimulated with a known agonist of PAR-2 (PAR-2 peptide SLIGRL-NH_2). Previous studies have shown that A549 cell line expresses the PAR-2 (Bohm *et al.*, 1996; Asokananthan *et al.*, 2002a) and human PAR-2 also stimulates with PAR-2 peptide and produces IL-8 (Larsen *et al.*, 2008).

To find out the increase in the production of IL-8 compared to untreated cells, "untreated cells" (write side) were stimulated with serum free medium while "SLIGRL-NH_2" (left side) were stimulated with PAR-2 peptide. In our study we have seen that the cells stimulated by PAR-2 peptide have shown more than 5 times more production of IL-8 compared to untreated cells as shown in the Fig. 3.4.

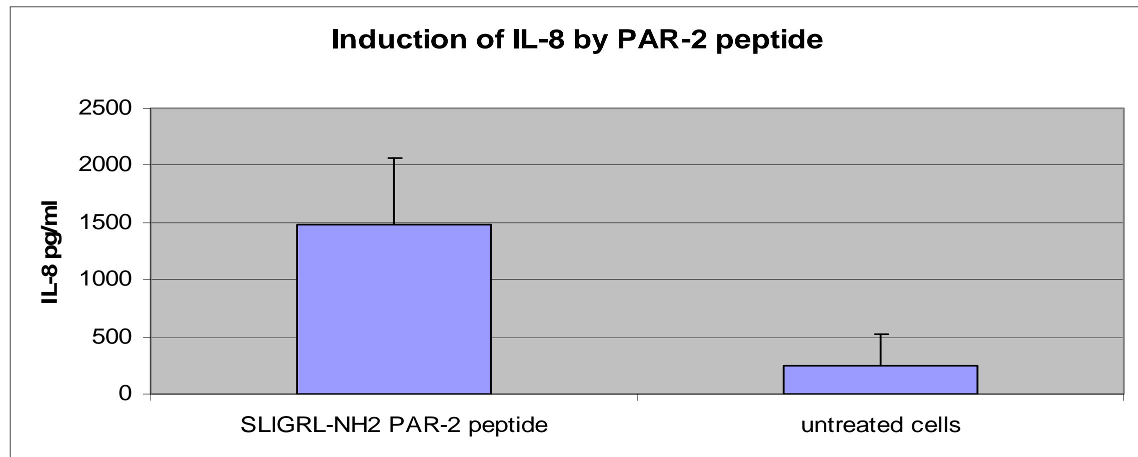

Fig.3.4 *Effect of PAR-2 stimulation on the secretion of IL-8 in A549 cells. A549 cells (1.5×10^5 cells/well) were seeded out in 24 well plates. On the next day the medium was changed. On the following day the cells were incubated in a serum free medium over a night. On the following day the cells were stimulated with PAR-2 peptide (SLIGRL-NH_2). The supernatant was harvested 6 hours later and was analyzed for the presence of IL-8 by ELISA. Data are expressed as mean±SD,($n = 3$).*

3.4.2. Effect of transfection of A549 cells on the production of IL-8

In order to determine the effect of transfection on production of IL-8, A549 cells were transfected with siRNA silencer (negative control) and then stimulated with PAR-2 peptide as shown in the Fig. 3.5. After transfection of A549 cells with negative control there was 17.5% reduction in the production of IL-8 compared to non transfected cells stimulated with PAR-2 peptide.

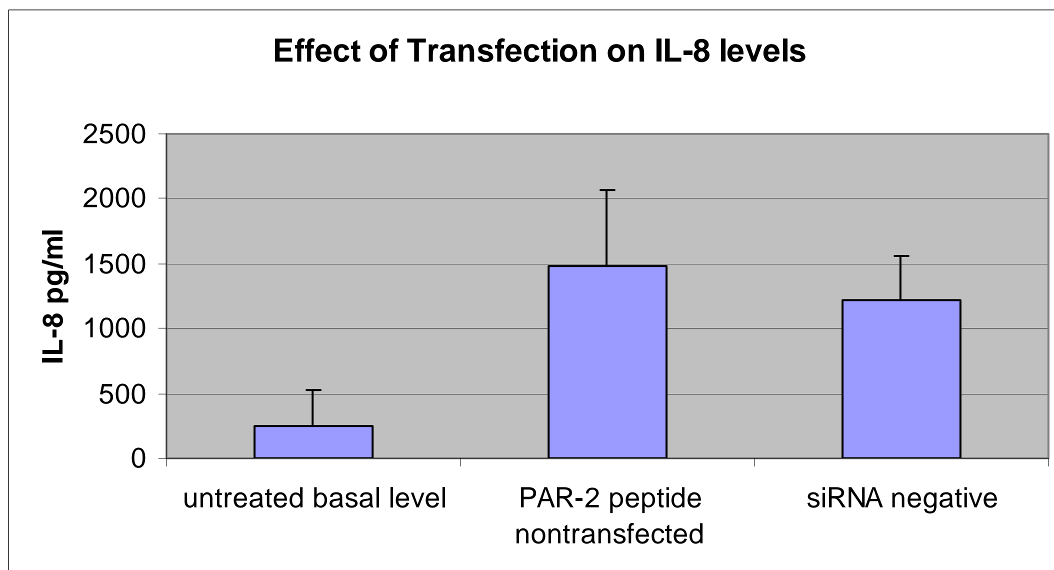

Fig.3.5 *Effect of transfection of A549 cells against PAR-2 peptide on the production of IL-8. A549 cells were seeded in DMEM/F12 in 24 well plates. 48 hour later the cells were transfected with siRNA negative control. Non-transfected, transfected and untreated cells were kept in serum free standard medium for 24 hours until the stimulation by PAR-2 peptide. 6 hours later the supernatant was harvested and was analyzed for the presence of IL-8 by ELISA. Data expressed as mean ± SD,(n = 3).*

3.4.3. PAR-2 involvement on the production of IL-8 by Moricrase stimulation.

To determine if moricrase induced IL-8 secretion in the A549 cells through activation of PAR-2, we have used a combination of the three siRNAs to knock down the expression of the PAR-2. As shown in the Fig. 3.6 there is 85.5% reduction in the production of IL-8 in PAR-2-siRNA transfected cells, compared to the cells transfected by the siRNA silencer (negative control). The amount of IL-8 production in PAR-2-siRNA transfected cells was 8.8% above basal level while the amount of IL-8 produced by A549 cells transfected with siRNA negative control was 60.65% above basal level.

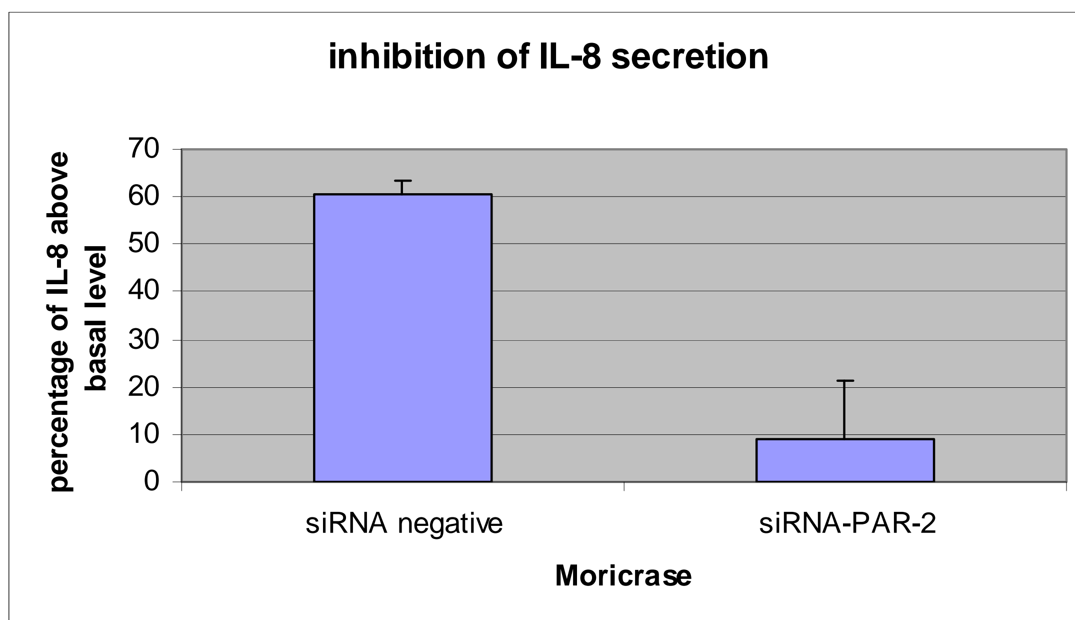

Fig.3.6 *Inhibition of IL-8 secretion from A549 cells using siRNA against PAR-2. A549 cells (1.5 × 10^5 cells/well) were seeded out in DMEM/F12, FBS and Glutamine in 24 well trays. Forty eight hours later the cells were transfected with a mixture of three different siRNAs targeting PAR-2 and with siRNA negative control. Cells were kept in serum free medium for 24 hours until stimulation with moricrase. The supernatant was harvested six hours later and was analyzed for the presence of IL-8 by ELISA. Data expressed as mean ± SD, (n = 2).*

3.4.4. Comparison of moricrase and spotted mackerel trypsin in stimulation of A549 cells and IL-8.

To determine the effect of spotted mackerel trypsin stimulation and production of IL-8 by A549 cells a comparison study have been performed with moricrase and spotted mackerel. A549 cells were stimulated with 0.01 mU/ml of spotted mackerel trypsin and 0.03325 µg/ml of moricrase as shown in the Fig. 3.7. The results have shown more than 6 times increase in the production of IL-8 by spotted mackerel trypsin in comparison with moricrase. The basal level shows non stimulated cells.

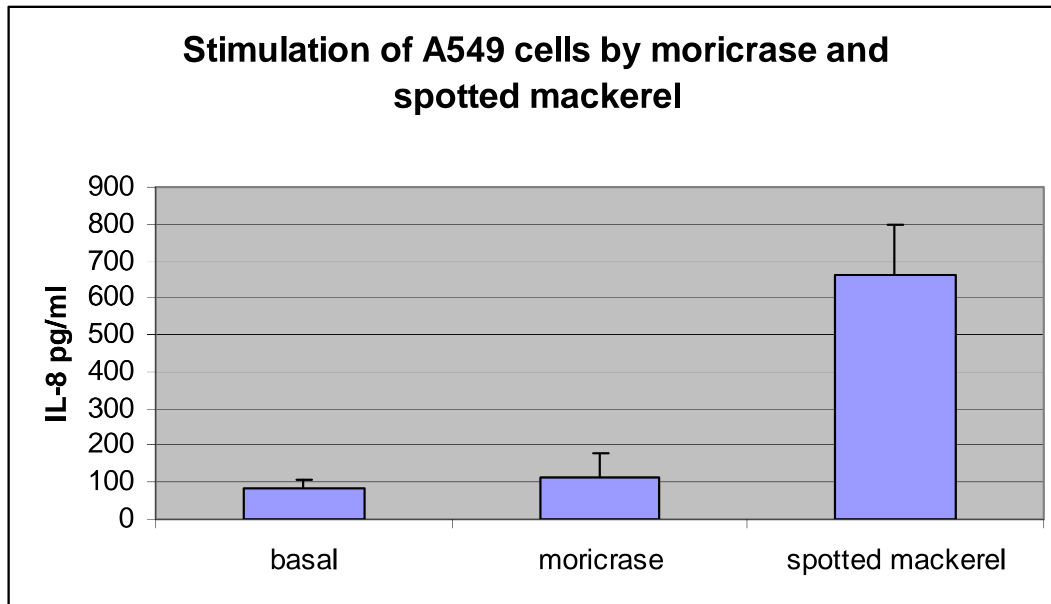

Fig.3.7 *Comparison between stimulation of A549 cells by moricrase and by spotted mackerel. A549 cells were seeded in DMEM/F12 in two different 24 well trays. 24 hour later the fresh medium were given to the cells after next 24 hours the cells were washed 3 times with DMEM/F12 and incubated over a night. The next day after the cells were stimulated with 0.03325 µg/ml of moricrase and 0.01mU/ml of spotted mackerel trypsin. 6 hours later the supernatant was harvested and was analyzed for the presence of IL-8 by ELISA. Data expressed as mean ± SD, (n = 3).*

3.4.5. Comparison of moricrase and spotted mackerel trypsin in inhibition of IL-8 stimulation by PAR-2 siRNA.

In order to compare the effect of siRNA PAR-2 on the production of IL-8 in moricrase and in spotted mackerel trypsin these studies were carried out as shown in the Fig. 3.8. Spotted mackerel trypsin at the concentration of 0.01 mU/ml was able to produce approximately 10 times more stimulation of IL-8, compared to 0.03325 µg/ml of moricrase while siRNA-PAR-2 is reducing the IL-8 levels for both stimulators.

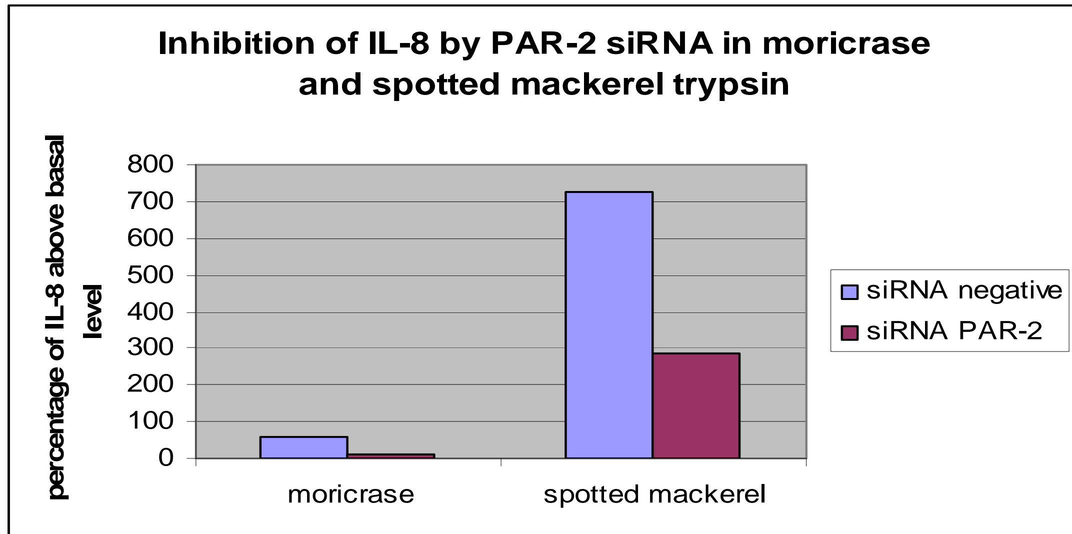

Fig.3.8 *Inhibition of IL-8 production by siRNA PAR-2 in moricrase and spotted mackerel. A549 cells (1.5 × 10^5 cells/well) were seeded out in DMEM/F12 in 24 well plates. 48 hours later the cells were transfected with a mixture of three different siRNAs targeting PAR-2 and with siRNA negative control. Cells were kept in serum free medium for 24 hours until stimulation with moricrase and spotted mackerel. The supernatant was harvested six hours later and was analyzed for the presence of IL-8 by ELISA. Data expressed as mean ± SD, (n = 2).*

4. Discussion

4.1. Usage of A549 cells in our experiments

A549 cells are human lung adenocarcinoma cells. A549 cells are not fully representative of normal human respiratory epithelium but have been a useful *in vitro* model to study human pulmonary epithelium (Wong *et al.*, 1998). A549 cell line has many characteristics in common with the human type II lung epithelial cells. The A549 cell line produces IL-8 after stimulation with a different variety of biological and chemical agents (Hansen, 1997; Van Wetering *et al.*, 1997; Palmberg *et al.*, 1998). These cells were used in all the experiments. A549 cells in our laboratory has shown clear response regarding production of IL-8 and these cells are highly sensitive against stimulation.

The cell culture of A549 was established in 1972. Growth curve experiments as shown in *Fig 3.1* (a, b, c) has followed the common growth curve behavior in our laboratory. Our growth curve experiment data depicted that the cell line behavior was not similar in all the experiments although A549 cell line is one of the well established model system. In the cultured cells, in general there may be a gradual change in the behavior that might be due to some small differences in growth conditions like media, supplements, antibiotics, temperature etc. that may favor a certain sub population of the cell line.

The documented doubling time provided by the supplier was 22 hours but in our lab experiments the mean doubling time was 30.33 hours. That might be due to favorable growth of subclasses generated by many replications. Doubling time up to 25 hours has been reported previously (Viale *et al.*, 2008). This reflects that the cell line is not showing the exact behavior all the time it might be due to some handling deficiencies but all the experiments were tried to be performed in the same conditions.

4.2. Moricrase as a stimulator of IL-8 levels in A549 cells.

The production of IL-8 at basal level (in a plane medium) increases gradually as the previous studies shows that A549 cell line produces IL-8 by itself in a time dependent manner (Arenberg *et al.*, 1996). There is only 58% increase in the production of IL-8 above basal level after six hours which leads to 70% increase in the production of IL-8 after 18 hours. The maximum production of IL-8 have been seen after 24 hours incubation, while after 48 hours the basal level of IL-8 was equal to the stimulation levels of IL-8 which shows exactly the same behavior as mentioned above that the cells produce IL-8 in the plane medium.

The moricrase concentration of 0.03325 μg/ml has shown maximum response while higher concentrations were not been able to produce better responses. Instead of moricrase other known stimulators of A549 cells like trypsin, have shown higher levels of IL-8 from A549 cells after 24 hours incubation (Sachse *et al.*, 2006). Endotoxins derived from P.Aeruginosa, produced more than 6000 pg/ml of IL-8 in A549 cells after 24 hours incubation in a dose dependent manner (Hansen *et al.*, 1999).

A possible reason for low stimulation of IL-8 by higher concentrations of moricrase might be due to internalization of PAR-2. The higher concentrations of moricrase might induce internalization or desensitization of PAR-2 by independent phosphorylation of the receptor at intracellular tail.

Morphological studies have shown the cell behavior after stimulation with different concentrations of moricrase which showed us that the higher concentrations of moricrase can cause detachment of the cells. We have studied the effect of trypsin on A549 cells and previous studies shows that trypsin is a power full cell dissociating agent and we have examined that the higher concentrations of moricrase leaded to cell detachment which may be the effect of trypsin.

Our results confirm that moricrase is a stimulator of IL-8 in A549 cells but has shown a difference in the stimulation compared to the known stimulators (trypsin, endotoxins). In short, we have an effective model system of cell responding to moricrase by IL-8 stimulation. In our three parallel experiments the difference between productions of IL-8 can be due to:

- **The cell concentration**. The cells were counted on Burker chamber as there is not any exact method of counting such a large number of cells. It was assumed that the number of cells were 150,000 per well by making the dilution of calculated cell suspension.
- **Differences in the cell treatment**. If the cells were less confluent before seeding or in low healthy condition they may need longer time to get in logarithmic cell growth and to produce maximum response to stimulation.

4.3. Possible consequences of IL-8 stimulation in vivo

IL-8 is an indicator of innate pulmonary inflammation; many studies have shown that A549 cells have the potential to produce IL-8 after stimulation (Hansen, 1997). The results have shown that moricrase is able to stimulate the production of IL-8 from A549 cells in our model system although it is difficult to compare *in vitro* results directly with *in vivo* situation. However if we study the similarities happening *in vivo*, this could mean that:

- Hepatopancreatic enzymes from king crab may promote inflammation, if inhaled in a significant quantity at the king crab industry.
- Pharmaceutical usage of moricrase may be less applicable if inflammatory reactions take place in skin epithelium, when moricrase is applied for healing purposes.

4.4. Induction of IL-8 by PAR-2 peptide.

SLIGRL-NH_2 is a known PAR-2 peptide which stimulates PAR-2 in A549 cells and produces the response. Fig. 3.4 showed the comparison between the responses of A549 untreated cells (plane medium) and A549 cells treated with PAR-2 peptide. It confirmed that PAR-2 peptide was stimulating A549 cells while the difference between untreated cells and the cells stimulated by PAR-2 peptide was more than 5 times as PAR-2 peptide has shown 1480 pg/ml production of IL-8 while untreated cells have shown only 243.3 pg/ml production of IL-8. This experiment has also confirmed that unstimulated cells (basal level) were also producing IL-8.

4.5. Effect of transfection on production of IL-8 levels

We have used a method of transfection in order to observe the involvement of PAR-2. As shown in *Fig 3.5* that transfection by itself, also effects on the production of IL-8. We have seen 17.5% reduction in transfected cells compared to non-transfected cells while both (transfected and non-transfected) cells were stimulated with PAR-2 peptide.

siRNA negative is a negative control with sequences that do not target any gene product. This substance is essential for determining siRNA transfection efficiency and to control for the effects of siRNA delivery. In order to be more precise about the experiments all results were compared with siRNA negative (negative control) instead of basal or stimulated levels.

4.6. Involvement of Protease Activated Receptors in moricrase stimulation of IL-8

In A549 cells activation of PAR-2 induces release of IL-6 and IL-8 (Asokananthan *et al.*, 2002a). In the respiratory system PAR-2 is mainly considered as pro-inflammatory (Knight *et al.*, 2001). Previous research shows that production of IL-8 from A549 cells by salmon trypsin (Larsen *et al.*, 2008) and by dust mites (Asokananthan *et al.*, 2002b) is PAR-2 mediated.

We examined the involvement of PAR-2 in the production of IL-8 by stimulating the cells with moricrase as shown in the fig. 3.6. Moricrase has shown 85.5% reduction in the production of IL-8 after treating with combination of three specific siRNA as compared to the cells transfected with siRNA negative (negative control). This experiment confirmed the involvement of PAR-2 in the production of IL-8 by moricrase. Lower stimulation levels of IL-8 by moricrase might be due to:

- The involvement of any other pathway for IL-8 stimulation;
- The transfection by PAR-2 siRNA is incomplete;
- The difference of structural activity of moricrase trypsin compared to other trypsin.

4.7. Comparison of moricrase and spotted mackerel trypsin in stimulation of A549 cells and IL-8.

Spotted mackerel trypsin is a known stimulator of PAR-2 and produces IL-8 in a PAR-2 dependent pathway (A. Larsen-personal communication). Reason for comparing spotted mackerel with moricrase was to understand the difference in the production of IL-8 after stimulating A549 cells with a pure trypsin and a mixture of proteases. In moricrase there is not only trypsin which affects the IL-8 levels as other proteases may also

produce effects. Elastase is one of the proteases present in moricrase and might be able to produce disarming effect in the receptor, preventing activation by trypsin (Dulon *et al.*, 2003). Trypsin is taking part in the stimulation but other proteases may also produce effect. So elastase and possibly other enzymes (present in moricrase), (Rudenskaya *et al.*, 2000) might cleave the amino terminal exodomain of PAR-2 downstream at trypsin cleavage/activation site thus preventing the attachment of trypsin to the receptor and blocking the stimulation.

The result of Fig. 3.8. have shown that in spotted mackerel the difference between production of IL-8 by PAR-2 siRNA transfected cells (red bar) and siRNA negative (negative control) transfected cells (blue bar) is only 60.35% while at the same condition moricrase has shown 85.5% difference in both transfected cells. This shows that moricrase is showing the involvement of PAR-2 but is not been able to produce higher amount of IL-8 like spotted mackerel trypsin.

4.8. Infections

We have seen a sudden drop in the production of IL-8 in the time response experiment after 12 hours (red dot in Fig 3.3) it shows that after 12 hours the stimulation in basal level was equal to the stimulation by moricrase as the samples were stimulated in parallel wells it might be possible that in the 12 hours wells any unseen contamination or infection was generated which cannot be jugged under microscope because in the same plate all other samples were present and not any problem was seen in any other sample so it means media and our incubator conditions were fine.

The time response experiment was performed total 5 times but each time we have seen some infections problem that can be due to;

- Absence of antibiotic in our media
- Any bacterial or fungal growth in the incubator
- The presence of infectious substance in micropipette etc.

Several reasons can be generated for infections problems because with the same materials in 6 hours incubation we have not seen any infection. To investigate the infection in our cell line, mycoplasma test was performed (data not shown) and the results have not shown any signs of infection in the cell line. The infections were also checked under microscope every time before performing the experiments and were not found any infection.

5. Conclusion

Moricrase has the ability to produce an inflammatory reaction in human pulmonary epithelial cells via stimulation of interlukin-8 (IL-8). This production of IL-8 was mediated through PAR-2 pathway. Stimulation of IL-8 by moricrase is time and concentration dependent. Concentrations of moricrase higher than 0.03325 µg/ml reduces the IL-8 stimulation. Spotted mackerel trypsin is a better stimulator of IL-8 as compared to moricrase. Moricrase is a mixture of enzymes including trypsin, further research is needed to see the effect of other enzymes of moricrase on PARs and inflammation.

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