Mechanism of Corneal Epithelial Cells Death by Infection of *pseudomonas Aeruginosa*through Analysis Expression of Caspase-1, TNF-α, RIPK1, RIPK3, Caspase-3 in Rats Model

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Abstract

Background: *Pseudomonas aeruginosa* is one of the leading causes of severe keratitis that may cause in progressive inflammation with rapid onset, resulting inpermanent tissue destruction, then eventually, blindness and damage to corneal integrity. It is important to know the virulence factor of *Pseudomonas aeruginosa* mechanism and modes of regulation in corneal epithelial cells to prevent progressivity, increasing chance of recovery, and decreasing perforating corneal complication.

Objective: The aim of this study to investigate mechanism of corneal epithelial cell death by *Pseudomonas aeruginosa* infection through the analysis of expressions of caspase-1, TNFα, RIPK1, RIPK3, and caspase-3.

***Methods*:** The study design was randomized post test only with control group. Fifty three Wistar rats are divided into 2 groups, each of them with 6 treatments. One control group and one experimental group. In the experimental group, three epithelial abrasion was produced on the left cornea with a 26-gauge needle and inoculated with $2x10^6$ CFU/ml in 5µl of bacterial suspension, while in the control group only three epithelial abrasion. In both groups use waiting time 1 hour, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours after that theeyeball was enucleated and rat was terminated thus examine for histology and immunohistochemicalstaining examination.

Result: *Pseudomonas aeruginosa* was shownto cause increasing expression of caspase-1(p=0.006), TNFα (p=0.000), caspase-3 (p=0.001), and decreasing expression of RIPK3 (p=0.047). TNFα showed to cause increasing expression of RIPK1 (p=0.000),RIPK1 showed to cause increasing expression of RIPK3 (p=0.000), but TNFα showed not to cause change of expression of caspase-3 (p=0.141).

Conclusion: This study demonstrated infection of *Pseudomonas aeruginosa* in corneal rats model showed to cause increasing expression of caspase-1, TNFα, caspase-3, and decreasing expression of RIPK3,as prime biomarker in mechanism of corneal epithelial cell death.

Keyword*: Pseudomonas aeruginosa, corneal epithel, cell death, caspase-1, TNFα, RIPK1,RIPK3, caspase-3.*

Background

Pseudomonas aeruginosa is a common opportunistic bacterial pathogen causes infection at various body sites

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including the cornea, and is a leading cause of blinding keratitis worldwide. *Pseudomonas* keratitits is a rapidly progressive and destructive disease that can cause severe symptoms, it can lead to corneal perforation and melt, and result in the loss of vision.[1]*P aeruginosa* becomes more pathogenic in lens-related biofilms, in turn enabling enhanced binding to molecular receptors exposed on injured epithelial cells. Once adherent, bacteria will proliferate and invade the corneal stroma, often with the aid of bacteria-specific proteases. Reactive host inflammation begins with the expressionof various cytokines and chemokines, recruitment of inflammatory cells from the tears and limbal vessels, and subsequent secretion of matrix metalloproteinases leading to characteristic corneal necrosis.[2]

P aeruginosa, which causes a significant proportion of bacterial keratitis, is responsible for 6% to 39% of cases in the United States and 8% to 21% in South India,in Indonesia there were 25% *Pseudomonas* keratitis from 220 keratitits cases in Jakarta and 26 % from 53 % positive culture in Surabaya.[3-5] Pathogenesis in *P aeruginosa* is mediated by multiple bacterial virulence factors that facilitate adhesion and/or disrupt host cell signaling pathways while targeting the extracellular matrix.[6] The subsequent tissue damage, invasion, and dessimination of *P aeruginosa* are likely attributed to the many virulence factors its produce.[7] Corneas from infected eyes mouse showed all the classical signs of inflammation, including the presence of polymorphonuclear leukocytes and massive corneal edema. Bacteria in various stages of penetration into epithelial cells were observed at 8 h. Lipopolysaccharide (LPS) is a predominant component of the outer membrane of *P aeruginosa* and plays a prominent role in activation the host's innate (TLR4, NLRP1, NLRP2, and NLRP3) and adaptive immune responses.[8] Flagellar proteins have been shown to play critical roles in attachment, invasion, biofilm formation and the mediation of inflammatory responses.[9] Flagellin mediators the inflammatory response via the innate immune system, through its specific ineraction with a number of pattern recognition receptors (PRRs) of the host.[10] Flagellin is recognized by both TLR5 and NLRC4, however different amino acid residues of flagellin were critical for sensing by NLRC4 and TLR5.[11] T3SS (Type III Secretion System) of *P aeruginosa* delivers up to four cytotoxins ExoS, Exot,ExoU, and ExoY directly to host cells, it also involves a flagellum-basal-body related system for delivering proteins directly from the cytoplasma of *P aeruginosa* into cytosol of host cells.[7,12,13] Exotoxin A has been demonstrated to be involved in local tissue damage and invasion. Exotoxin A enters host cells by receptors-mediated endocytosis and catalize the ADP-rybosilation of eucaryotic elongation factor-2 (EF-2) that inhibits protein synthesis, which ultimately leading to ceellular death. Virulence factors of *P aeruginosa* and its mechanism in the cornea require further research because many questions are not yet answered, such as how and when the already discovered virulence factors are involved in the pathogenesis infection on the cornea. It is important to developed biological drugs other than antibiotics that are more effective using new approaches against currently existing infections.

Material and Methods

*Pseudomonas aeruginosa*wasobtained from corneal specimen keratitis patient in ophthalmology ward Dr. Soetomo Hospital Surabaya, cultured in microbiology laboratorium departement in theaforementioned hospital.

Animal models are 57 wistar rats (53 rats for experimental model and 4 rats for negative control) aging twelve-forteen weeks from Faculty of Veterinary Medicine Airlangga University Surabaya. The rats are anasthesized by intramuscular injection 0.3 ml/100gr body weight rat cocktail (ketamin 2ml,xylazine 1.25 ml, ACP 0.33 ml, saline 6.41 ml). After the rats had been anasthetized, three full thickness epithelial abrasions were produced on the left cornea with the 26-gauge needle. The corneas were divided into two groups. The first group was immediately inoculated with $2x10^6$ CFU/ ml *Pseudomonasaeruginosa* in the dose of 5 µl of bacterial suspension, while in the second group only epithelial abrasion without inoculated bacteria. At various times at 1 hour, 6 hours, 12 hours, 24 hours, 48 hours and 72 hours after inoculation, rats were terminated by cervical dislocationafter anasthetized previously and than enucleated.

Immunohistochemistry examination was done after eyeball tissue fixated in 10% buffer formalinfor

24 hours, excise cornea from eyeball then fixating, sectioning and staining with Meyer Hematoxylin, finally immunohistochemistry processes with monoclonal Ab caspase-1, TNFα, RIPK1, RIPK3, caspase-3 and prepare for examination with light microscope to show their expression.

Data were collected and processed with SPSS 23 software. Data were analyzed by MANOVA, and to explain death cells mechanisme use path analysis with regression.

Result

Clinical pictures of rats' cornea

All rats' corneas in control group (corneaabrasion only) at 1 hour, 6 hours, 12 hours,24 hours, 48 hours, and 72 hoursare clear. In treatment group (cornea abrasion+ *P aeruginosa* inoculation), rats' corneas at 1 hour and 6 hours after inoculation P aeruginosa are still clear, but at 12 hours there were opacity in the corneas, at 24 hours there were opacity extending, at 48 hours all corneas have abcess, and at 72 hours all corneas have abcess accompanied with corneal thinning.

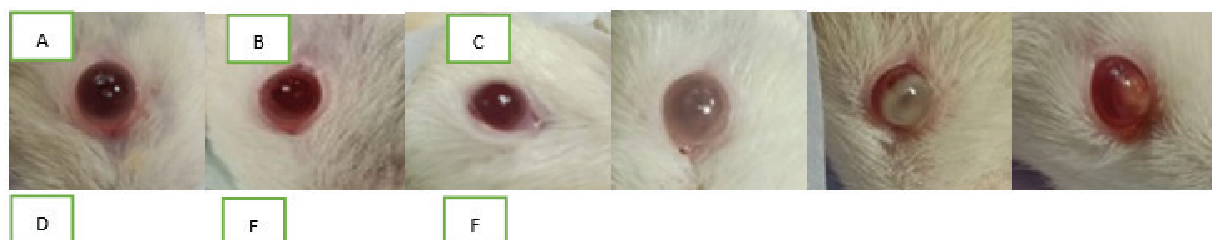

Figure 1 The group of rats' cornea abrasion + PA inoculation. A and B at 1 h and 6 h showed thatcorneasare still clear, C at 12 hshowed opaque in cornea,D at 24 jam showed spreadingcornea opacity, E at 48 hshowed wideningcorneal abcess, and F at 72 hshowedcorneal abcessandthinning.

Expressions of variables

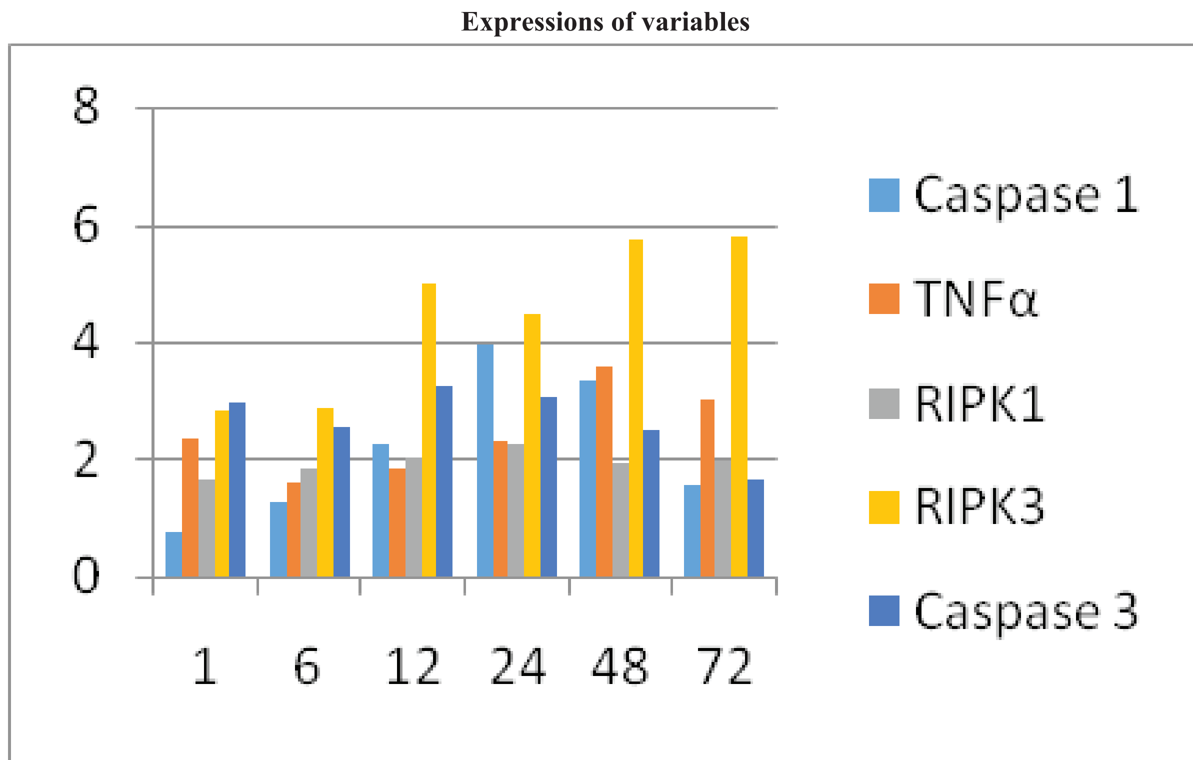

Figure 2 Expressions of variables after corneal rat's abrasion. The highest expression is shown by RIPK3, and the lowest by caspase-1.

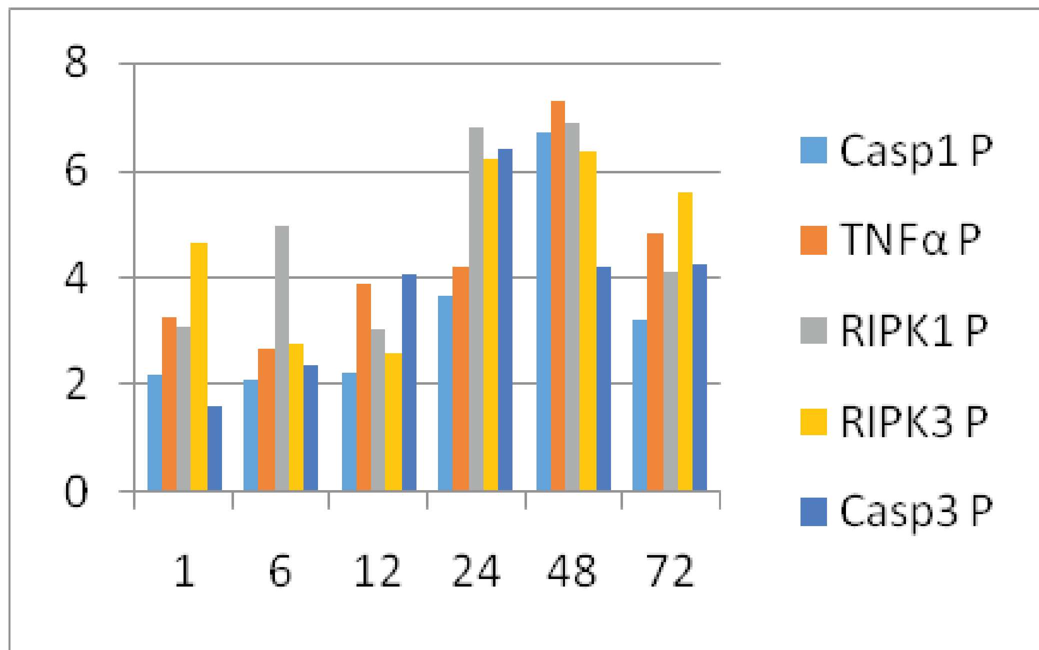

Figure 3: Expressions of variables after corneal rat's abrasion and inoculation P aeruginosa. The highest expression is shown by TNFα, and the lowest by caspase-3

Multivariate analysis

Table 1 Result of MANOVA analysis

Variables	Df	F	p
Caspase-1	12	12,120	0,000
TNFα	12	26,284	0,000
RIPK1	12	34,846	0,000
RIPK3	12	17,608	0,000
Caspase-3	12	19,362	0,000

Table 1 shows that RIPK1 has the most influence expression of variable, followed by TNFα, caspase-3, RIPK3, and caspase-1.

Table 2: Result of Path analysis

Affecting variables	Affected variables	B	p
P aeruginosa	Caspase-1	0,374	0,006
	TNFα	0,611	0,000
	RIPK3	0,381	0,047
	Caspase-3	0,445	0,001
TNFα	RIPK1	0,727	0,000
RIPK1	RIPK3	0,703	0,000

Table 2 shows that P aeruginosa increament of caspase-1, TNFα, caspase-3, decreament of RIPK3, TNFα increasing of RIPK1, and RIPK1 increasing RIPK3.

Discussion

The epithelium as the outer barrier is constantly self-renewing and has the highest regenerative capacity, as epithelial cells are replenished every 7-10 days. Epithelial stem cells reside in the limbal palisades and migrate towards the corneal center, where they differentiate to transient amplifying cells and basal cells.[14,15]Thus, renewal of epithelial cells not only involves a vertical movement of differentiating cells from deep to superficial layers, but also centripetal migration of stem cells from the limbus to the central cornea as they undergo differentiation.[15] Epithelial injury and apoptosis of the injuried cells leads to disruption of the attachment to the underlying basement membrane. Subsequently, cells from the wound margin rapidly respond with flattening and centripetal migration. As cell-to-cell adhesion are partially maintained, the defect is slowly covered by a sliding cellular sheet. ECM facilitates epithelial migration to cover the wound. Fibrin, fibronectin and hyaluronic acid are some of the molecules identified in this matrix.[16]In general, immune cells recruitment after corneal injury is mediated by proinflammatory cytokines released from epithelial cells, and keratocytes at the injury site. IL-1,IL-6 and TNFα have been shown to be important mediators.[17,18] Neutrophils are the first cells infiltrating the cornea after injury, they can be detected as soon as 2 h after injury and have been observed to enter the cornea in two major waves at 18 and 30 h after epithelial abrasion, as soon as 48 h after injury, their number normalize again.[19] Kalha S et alshowed at their study about corneal epithelial abrasion in murine model, 18 h post injury the healing process is active, ree-pithelialization is ongoing and the surface was fully re-epithelialized by 72 h after abrasion.[20]

Transmission electron microscopy has shown that Pseudomonas can infect stroma within one hour of adhering to an injured corneal epithelium. Within 6-8 hours, it produces grayish superficial epithelial and stromal microinfiltration with oedema at the edge of the injury. During the next 18-24 hours, the stromal infiltration extends horizontally and vertically. There is a severe anterior chamber reaction with hypopyon. The symmetric and concentric extension involves the whole width and depth of the cornea. There is a characteristic diffuse grayish, epithelial inflammation and infiltration away from the main corneal lesion. During the next 48-96 hours, if untreated, a ring infiltration develops with scleral and corneal melting associated with greenish yellow mucopurulent discharge adhering to the ulcer. Within 2-5 days, an untreated corneal ulcer may lead to descemetocele formation and perforation of the cornea.[21,22]

Expression of Caspase-1

The best described function of caspase-1 is its key role in the processing on inactive IL-β dan IL-18 into mature inflammatory cytokines. Aditionally, excessive caspase-1 activity can cause pyroptosis, a non-apoptotic type of programme cell death.[23] Caspase-1 is activated by dimerization at complexes termed inflammasomes that from in the cytosole and detect a diverse repertoir of pathogenic molecule, including bacterial toxins and viral RNA.[24] Inflammatory caspases (caspases 1, 4, 5 and 11) are activated in response to microbial infection and danger signals. When activated, they cleave mouse and human gasdermin D (GSDMD) after Asp276 and Asp275, respectively, to generate an N-terminal cleavage product (GSDMD-NT) that triggers inflammatory death (pyroptosis) and release of inflammatory cytokines such as interleukin-1β 1,2.[25,26] Since caspase-1 classically cleaves the pro-forms of IL-1beta and IL-18 to the mature active molecules, flow cytometry of cells in the infected cornea by P aeruginosa was done to investigate the effect of NLRC4 silencing on caspase-1 activation; McClelland et al.,2017 determined the effect of NLRC4 silencing on caspase-1 activation in CD45 expressing cells in the infected corneas at day 5 p.i.The results suggest that NLRC4 silencing reduces caspase-1 activation in $CD11b^{low}Ly6G^{low}$ cells, but not in conventional macrophages or neutrophil populations present in the *P. aeruginosa* infected corneas.These data provide evidence that the NLRC4 inflammasome contributes to resistance through regulation of caspase-1, IL-1beta and IL-18 in a $CD11b^{low}Ly6G^{low}$ population of cells.[27]

Expression of TNFα

Tumor necrosis factor (TNF) is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction.

It is produced chiefly by activated macrophages, although it can be produced by other cell types as well. The tumor necrosis factor (TNF) superfamily (TNFSF) and the TNF receptor (TNFR) superfamily (TNFRSF) form the corresponding ligand and receptor systems that are widely distributed in different tissues and cell types. Collectively they play critical roles in numerous aspects of mammalian biology, including embryonic development, innate and adaptive immunity, and maintenance of cellular homeostasis. TNFα has a wide range of biological effects in host defense against pathogens. On a cellular level, it is capable of inducing cell survival, proliferation, and differentiation, as well as both apoptotic and necrotic cell death under certain conditions.[28] TNFα not only promotes the production of inflammatory cytokines but also enhances the adhesion and permeability of endothelial cells and promotes the recruitment of immune cells such as neutrophils, monocytes, and lymphocytes to sites of inflammation.[29,30] While bacterial lipopolysaccharide (LPS) serves as a major stimulant of the innate immune system, microbial antigens, enterotoxins, and cytokines including TNFα itself are also able to trigger TNFα production. TNFα also stimulates the generation of numerous pro-inflammatory cytokines including IL-6, IL-8, TNFα itself, adhesive molecules, chemokines, and metalloproteinases, potentially leading to a TNFα-mediatedpro-inflammatory autocrine loop.[31-33] Chao in his research in corneal wistar rat's showes At 1 h after phototherapeutic keratectomy (PTK), the gene expression of the cytokines TNF-a and IL-6 was higher than in untreated controls, but lower than 12 h after treatment (Table 1). The increases observed between 1 and 12 h after PTK were statistically significant for both cytokines (P ¼ 0.0005 and P ¼ 0.0078, respectively. The expression of the inflammatory cytokines TNF-a and IL-6 was detected not only in epithelial, endothelial, and infiltrating cells,4 but also in the keratocytes from the corneal stroma. Whereas at 24 h after PTK, thexpression of both cytokines remained higher than in the controls. Four highly homologous mammalian family members have been identified: TNFAIP8, TIPE1 (TNFAIP8L1), TIPE2 (TNFAIP8L2), and TIPE3 (TNFAIP8L3). TNFAIP8 expression is induced by TNFα.[34] The present study has demonstrated that TIPE2−/− increased the susceptibility of mouse corneas to *P aeruginosa* infection via enhancing NF-κB signaling and the infiltration of inflammatory cells. It suggested a previously unrecognized role for TIPE2 signaling in limiting bacterial keratitis, and it also suggested that delivery of TIPE2 might be a potential therapeutic approach for corneal infection.[35]

Expression of RIPK1

Recent studies have implicated the intracellular signaling kinase RIP1 as a key switch of cell fate regulation. Depending on the cellular context, RIP1 controls whether the pleiotropic cytokine TNF induces NF-κB activation, apoptosis, or programmed necrosis.[36] When RIP1 ubiquitination is blocked by removal of the E3 ligases cIAP1 and cIAP2 through genetic ablation, RNA interference (RNAi) knockdown, or inhibitor of apoptosis (IAP) antagonists, RIP1 forms a secondary complex in the cytosol with Fas-associated death domain (FADD) and caspase-8-termed the Ripoptosome—to initiate apoptotic cell death.[37-39] Active caspase-8 within the Ripoptosome cleaves and inactivates RIP1and RIP3.[40-42] When caspases are inhibited by pharmacological inhibitors or under certain physiological conditions such as viral infections, RIP1 and RIP3 form the necrosome to initiate a third pathway known as programmed necrosis or necroptosis.[44,45]While more recent data further support inhibition of RIPK1 kinase as a therapeutic target, it also suggests that the functions of the kinase activity of RIPK1 in disease are not limited just to necroptosis. Several lines of evidence suggest that kinase activity of RIPK1 can also exert direct cell intrinsic controls on pro-inflammatory gene expression and, thus, promote inflammation independently of the DAMPs released as a result of necroptosis.[46]

Expression of RIPK3

hhRIPK3 is brought into the complex through interaction between homologous RIPK1 and RIPK3 RIP homotypic interaction motif (RHIM) domains, which promote formation of detergent-insoluble amyloid-like structures.[19] In necrosomes, RIPK3 undergoes posphorylation on Ser232, which is essential for recruitment of downstream necroptosis effector – pseudokinase MLKL.[47,48] MLKL is the critical necroptosis effector, as Mlkl -/- cells are completely

resistant to necroptosis.[49] Notably, the main function of necrosome formation in necroptosis initiation may be to promote formation of RIPK3 homodimers, as enforced homodimerization of just RIPK3 is sufficient for necroptosis even in the absence of interaction with RIPK1.[50-52] MLKL is directly phosphorylated by RIPK3 on Thr357/ Ser358 sites in the activation loop, inducing a conformational change that releases the pronecrotic membrane-disrupting N-terminal four-helix bundle.[53] MLKL is a gateway for activation of RIPK3 kinase-dependent necroptosis.[54,55] Plasma membrane translocation of MLKL has been linked to perturbation of calcium and sodium fluxes, which is associated with increased osmotic pressure.[56-58] Furthermore, calcium influx through TRPM7 channel was shown to be an early and essential eventin MLKL-dependent necroptosis. MLKL-dependent membrane permeabilization can be induced by enforced dimerization of just the N-terminal bundle and brace domains, an involves interactions with phosphoinositides.[54,58,59] However, the precise mechanism of how this ultimate step in necroptosis execution occurs is still a matter of discussion.[60]

Expression of caspase-3

Caspase-3 is one of the effector caspases that is central in executing the apoptotic process. To determine whether caspase-3 is involved in Pseudomonas aeruginosa Exotoxin A (ETA)-induced apoptosis of mast cells, several approaches were taken. Western blotting analysis using a mAb that specifically recognizes the activated form of caspase-3 demonstrated that ETA treatment induced activation of caspase-3 in mast cells in a dose-dependent manner. ETA-induced mast cell apoptosis is dependent on caspase activation. Human mast cells (HMC-1) were treated with 100 M Z-VADfmk for 1 h prior to a challenge with 300 ng/ml ETA for 18 h. Cells were analyzed for ssDNA using flow cytometry. To further examine the population of caspase-3-positive cells after ETA treatment, flow cytometry analysis was carried out using intracellular staining with a FITC-labeled mAb specific for the activated caspase-3. ETA treatment of HMC-1 cells induced a dose-dependent increase of active caspase-3-positive cells. Approximately 22% of the HMC-1 cells were stained positive for the active caspase-3. *Pseudomonas* exotoxin A (PE) is a bacterial toxin that arrests protein synthesis and induces apoptosis. Du et al utilized mouse embryo fibroblasts (MEFs) deficient in Bak and Bax to determine the roles of these proteins in cell death induced by PE. PE induced a rapid and dose-dependent induction of apoptosis in wild-type (WT) and Bax knockout ($Bax^{-/-}$) MEFs but failed in Bak knockout ($Bak^{-/-}$) and Bax/Bak double-knockout (DKO) MEFs. Also a loss of mitochondrial membrane potential was observed in WT and $Bax^{-/-}$ MEFs, but not in $Bak^{-/-}$ or in DKO MEFs, indicating an effect of PE on mitochondrial permeability. PE-mediated inhibition of protein synthesis was identical in all 4 cell lines, indicating that differences in killing were due to steps after the ADP-ribosylation of EF2. Mcl-1, but not Bcl-x_L, was rapidly degraded after PE treatment, consistent with a role for Mcl-1 in the PE death pathway. Bak was associated with Mcl-1 and Bcl-x_L in MEFs and uncoupled from suppressed complexes after PE treatment. Overexpression of Mcl-1 and Bcl-x_L inhibited PE-induced MEF death. The data suggest that Bak is the preferential mediator of PE-mediated apoptosis and that the rapid degradation of Mcl-1 unleashes Bak to activate apoptosis.Zhang J et al (2004) showed increase in the number of apoptotic cells, in elevated cellular caspase-3 activity, and/or in increased cleaved poly (ADP-ribose) polymerase in P. aeruginosa-infected on primary culture of human corneal epithelial cells.[61]

The normal rat's cornea with the greatest expression of variable is RIPK3 with the mean value of 1.55. Rat's corneaafter abrasionhas RIPK3 as the greatest expression of variable with mean value of 5.8 at 72 h (figure 5.21). Receptor interacting protein kinase is essential serine/threonine kinase for necroptosis, a type of regulated necrosis. Activated RIPK3 in turn phosphorylates and activates the downstream necroptosis executioner mixed lineage kinase domain-like (MLKL).[40] However, recent evidence indicates that RIPK3 also exhibits phenotypes in *Ripk3*$^{-/-}$mice could at least in part be attributed to these non-necroptotic signaling functions.[62] RIPK3 also carries a unique homotypic protein-protein interaction domain, called RIP homotypic interaction motif (RHIM) at the carboxy terminus. In the quiescent state, the kinase domain of RIPK3 masks and prevents the RHIM for polymerization. In addition to phosphorylation,

RIPK3 undergoes K48-linked polyubiquitination in the kinase domain during normal turn over of the protein. Normally, this event does not lead to necroptosis as the unmasked RIPK3 rapidly degraded by proteasome. However, inhibition of the proteasome can trigger receptor –independent necroptosis.[62] Their study can explain the height of RIPK3 after corneal abrasion in this study. Post abrassion and bacteria inoculation on the rat's cornea, the greatest expression of caspase-1 at 48 h with mean value of 6.7, TNFα at 48 h with mean value of 7.2, RIPK1 at 48 h with mean value of 6.3, RIPK3 at 48 h with mean value of 6.3, and caspase-3 at 24 h with mean value of 6.4 (figure 5.22). The greatest expression of caspase-1, TNFα, RIPK1, and RIPK3 at the same time is in 48 h, indicated that the time of the greatest of cell death at 48 h.

Conclusion

In this research, *Pseudomonas aeruginosa* infection on the lab rats after 1 h, 6 h, 12 h, 24 h, 48 hand 72 hshow thecorneal epithelial cell death mechanismswhich result in theincrementof caspase-1, TNFα, and caspase-3 expressions, the decrement of RIPK3 expression,increment of RIPK1 caused by TNFα, but TNFα which does notincrease caspase-expressioncompletes theprevious researchesabout pathophysiology ofcorneal inflammationcaused by*Pseudomonas aeruginosa*infection in biomolecular study. New discoveries obtained from this researchare the fact that there is a decrement of RIPK3 caused by*Pseudomonas aeruginosa*and TNFα that does not cause the increment of caspase-3 expression.

Conflict of Interest: The authors declare no conflict of interest.

Ethical Clearance: This study had been approved by Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.

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