CHAPTER V

DISCUSSION AND CONCLUSION

The present study aimed at identifying potential virulence factors involving in brain infection from *M. pneumoniae* genome. P1 adhesin and enolase were identified by genome analysis as putative virulence factors. They were then characterized structurally. P1 adhesin is composed of 1,627 amino acids. However, a suitable template for P1 adhesin modeling could not be identified since amino acid sequence identity between P1 adhesin and that of a protein with known structure was too low (16%) to obtain good quality model by comparative modeling.

The MpnE model consists of two main domains, the smaller is the N-terminal and the larger is the C-terminal domain. The catalytic site and conformation of 8 α/β anti-parallel strands of TIM barrel are conserved in the predicted MpnE protein. Notably this model represented particular variant loop from residue 264 to residue 276. Additionally, this investigation shows that implicit solvent models and MD simulation were helpful to improve the model quality as shown in Tables 5 and 6.

Five hundred complex formations from docking experiments with MpnE and hPg by Hex 4.5 program were carried out to obtain the best fit that showed the same features of interaction site of complexes in each round of docking between MpnE and hPg. The optimal model of the complex was selected by lowest total binding energy and the experimentally determined epitope on the electrostatic surface correlation type (Wang *et al.*, 1998; Wu *et al.*, 1991) of MpnE and hPg implemented by Hex 4.5 program. In order to choose the most appropriate model MpnE-hPg complexes were further analyzed by MD simulation. Subsequently the amino acid side chain of interaction between MpnE and human plasminogen was also analyzed. The hydrogen bond interactions were then joined and visualized using the Chimera program. This study showed the complex interface produced 7 hydrogen bonds. The selected MpnE-hPg complex and the decrease of ASA of each residue that form complex were observed by *WHATIF* and Chimera program. This result showed the strongest interaction between MpnE-hPg complex in the contribution of hydrogen bonding and decreasing in ASA between MpnE and hPg. Our complex model of the MpnE-hPg is

in agreement with the study of LBS of plasminogen proposed previously in terms of the characteristic of charges and well conserved hydrophobic pairing would be allowed in our model. It has been known that LBS, contains cationic, anionic, and lipophilic areas, which interact with electrostatics, hydrophobic, and steric requirements of lysyl-binding type (Ehinger *et al.*, 2004; Gong *et al.*, 2001). This study agreed with the finding mentioned above by which residue Glu21, Asp26, and Asp67 are located in the LBS anionic center. These residues interacted with MpnE on the region with cationic charge, Lys and Arg, at the opposite end of the binding pocket configured by rendered side chains of residues. The trivial cavity of plasminogen for ligand binding is composed of Gln28, Pro30, Pro68, Tyr50, and Thr 66 (Figure 12A). Side-chain rings of residue Pro30, Pro68, Tyr50 conforms a lipophilic platform that lines the LBS. Pro30, Pro68 are a part of the hydrophobic core subjacent the LBS and Tyr 50 neighbors the LBS anionic end.

It has been reported that *M. pneumoniae* involves in neurological disorders (Sotgio *et al.*, 2003). Although there may be several steps related to invasion, the mechanism of this process remains unclear. In order to appear in the CNS and cause brain disease, circulating *M. pneumoniae* has to cross the BBB. It has been shown that *C. albican*, in *in vitro* model, has ability to penetrate HBMEC which is facilitated by its membrane enolase-human plasminogen interaction in the presence of tPA (Jong *et al.*, 2003). Based on the result of this study it is reasonable to propose that interaction between MpnE-hPg may play an important role for *M. pneumoniae* invasion across human brain. *M. pneumoniae* has been assigned to be a surface parasite as it is able to attach onto the surface of epithelial cells observed by autoradiography and transmission electron micrograph (Hu *et al.*, 1977). Generally, there have been several mechanisms for bacterial invasion. It has been reported that the mechanism of *M. fermentans* and *M. pneumoniae* invasion were non-phagocytic strategy (Yavlovich *et al.*, 2004). It has been known that membrane associated proteolytic activities of streptokinase from *Streptococcus* species and staphylokinase from *Staphylococcus* species are involved in invasion by interacting with host plasminogen (Loy *et al.*, 2001; Molkanen *et al.*, 2002). The interaction of bacteria with the plasminogen system which have been recognized as a mechanism possibly involving in bacterial invasion into host cell were observed in several pathogens such as *E. coli* (Leytus *et al.*, 1981), *Mycobacterium tuberculosis* (Monroy *et al.*,2000),

Yersinia pestis (Sodeinde *et al.*, 1992), *Staphylococcus aureus* (Molkanen *et al.*, 2002).

Additionally, *M. fermentans* which was able to bind plasminogen has been recently observed. The bound plasminogen was activated by urokinase to plasmin, consequently increasing of invasive capacity (Yavlovich *et al.*, 2004). Notably, it has been increasingly recognized that α-enolase is an important mediator of tissue pathology in infectious diseases (Redlitz *et al.*, 1995). Logically *M. pneumoniae* may use enolase-plasminogen interaction system for invasion of host, based on the above findings and the results of this study (Figure 14).

Plasminogen is a key component of fibrinolytic system and is inactive by itself. The plasminogen activation can be mediated either by tissue-type plasminogen activator (tPA) or by bacteria-origin activator such as streptokinase. The example is a 414-residue protein from *Streptococci,* streptokinase (Wang *et al.*, 1998). This protein is basically not proteases, but forms a tight-binding stoichiometric complex with human plasminogen or plasmin (Loy *et al.*, 2001). Moreover, such report showed the binding of streptokinase on to plasminogen activates and converts it to plasmin. The kringle domains of plasminogen which contain lysine binding site (LBS) have been characterized by chemical modification (Hochschwender and Laursen, 1981), NMR spectroscopy (Rajante *et al.*, 1994), and X-ray crystallography (Wu *et al.*, 1991). The activation of plasminogen to plasmin at the surface of cell membranes is a specific and complicated scheme leading to extracellular proteolysis implicated in a several biological functions, cell migration and extracellular matrix degradation. Plasminogen interacts with activator, tPA, at specific binding sites inducing conformational changes that make it susceptible to the cleavage of plasminogen to an active form, plasmin. In addition MpnE-hPg complex may favor *M. pneumoniae* dissemination and penetration into BBB endothelial cell causing host cell invasion.

There have been relevant evidences of several pathogens crossing HBMEC facilitated by binding between microbial α-enolase surfaces protein and human plasminogen in the presence of tPA or uPA (Rios-Steiner *et al.*, 2001; Jong *et al.*, 2003; Yavlovich *et al.*, 2001). It has been clearly known that plasmin is a serine protease with wide substrate specificity which can cleave complement factors, connective tissue protein, and component of basement membranes (Ogston, 1998). It has been proposed that bacteria bound-plasminogen enhances tPA to cleave arginine-

valine of plasminogen and activates it to plasmin. It is reasonable to propose that plasminogen bound to MpnE might enhance tPA and cleave arginine-valine to activate plasminogen to plasmin. Thus, it would be involved in the activation of mechanism that generated a targeted, localized including transient proteolytic activity. In addition, plasmin accumulated to this organism may therefore inactivate chemoattractants and thereby modulating tissue tropism. Such phemonena were observed in *Yersinia pestis* (Sodeinde *et al.*, 1992) and *Streptococcal* species (Pancholi *et al.*, 1998). It has been generally known that binding of plasmin to its receptor on cell surface would lead to releasing of cytokine and chemokine and initiate pro-inflammatory process (Syrovets *et al.*, 2001). The increase of cytokine authorizes endothelial cell remodeling, leading to impairment of barrier integrity (Geelen *et al.*, 1993) that may well facilitate *M. pneumoniae* invasion (Figure 15).

In such case plasmin accumulation would induce endothelial cell detachment (Reijerkek *et al.*, 2003). In addition an occurrence of fibrin mesh and blood vessel breakdown resulted from inflammatory process promoting host invasion was also described in *M. tuberculosis* invasion system (Monroy *et al.*, 2000). In order to test whether plasminogen can associate with *M. pneumoniae*, this dissertation has reported that *M. pneumoniae* bound to plasminogen facilitated by its surface enolase. Though it would confirm this evidence in detail by established experimental laboratory to observe the expression of MpnE on its cell surface by immuno-histochemistry assay which then be viewed enolase distribute on cell surface by transmission electron micrograph. Then, *in vitro* model for transcytosis may be observed through the transwell experiment in the presence or absence of plasminogen and plasminogen activator.

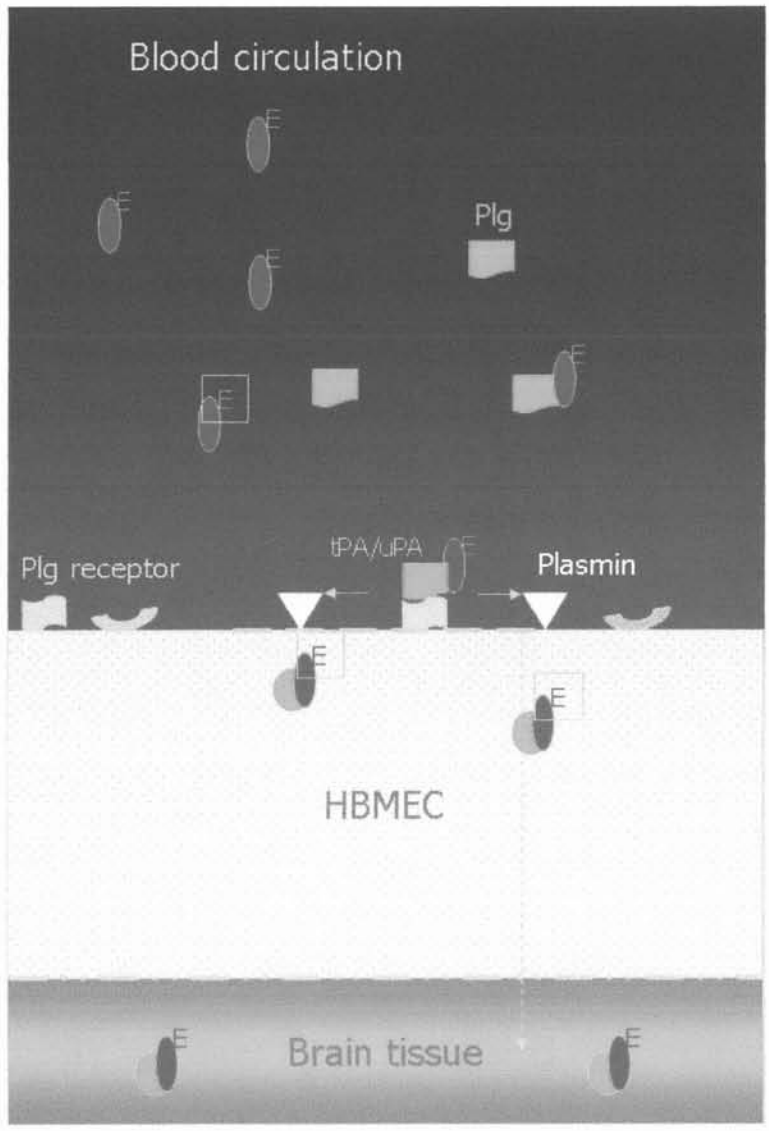

Figure 14 Proposed mechanism of *M. pneumoniae* involving in human brain invasion. *M. pneumoniae* interacts with human plasminogen by its cell surface enolase.

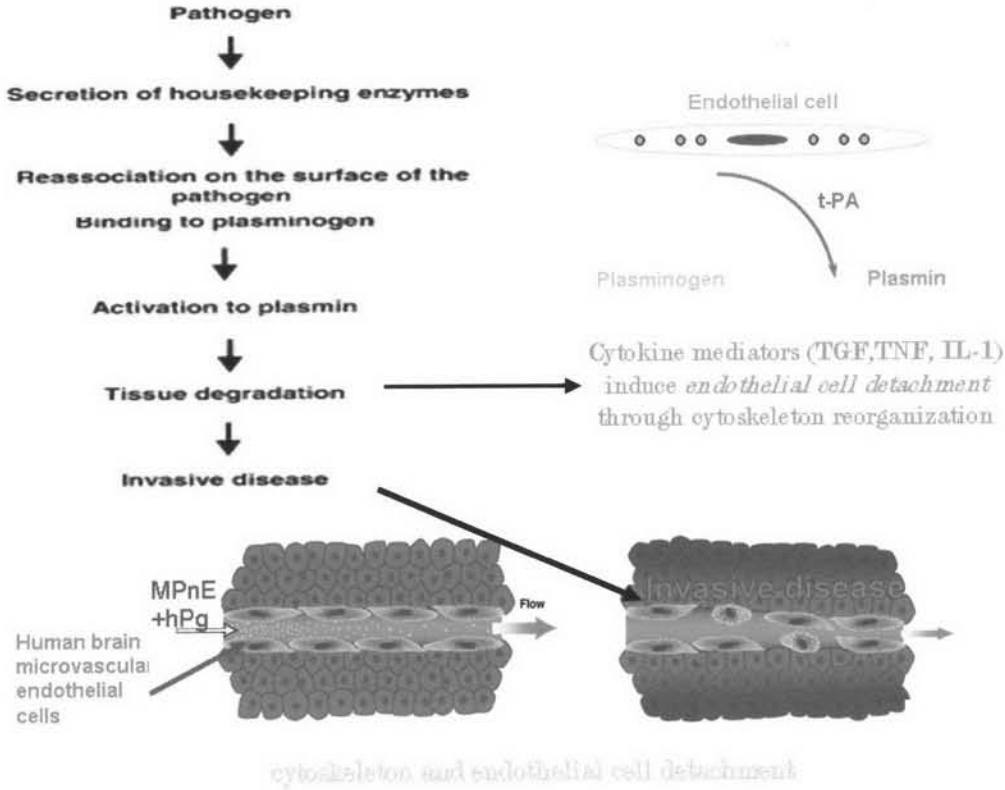

Figure 15 Plasmin augments the releasing of cytokine that is involved in endothelial cell dysfunction. An accumulation of plasmin on cell surface leads to endothelial cell detachment mediated by a function of cytokines i.e. TGF, TNF, and IL-1. Thus this occurrence may well promote *M. pneumoniae* invasion of human brain

Conclusion

In conclusion, this study reports the binding affinity of MpnE-human plasminogen kringle domain 2 on structural basis. Currently, the molecular basis by which *M. pneumoniae* invades and stably infect human cells, particularly CNS, remains largely elusive. A number of nonmycoplasmal pathogens are known to become pathogenic by using the host plasminogen system (Coleman and Benach, 1999). This dissertation proposed that the ability to invade human brain of *M. pneumoniae* mediated by plasminogen activation, effected on endothelial cell dysfunction. This was also supported by data of others as mentioned in the Discussion. Binding of MpnE to plasminogen and subsequent activation by plasminogen activator are a promissing virulence determinant of *M. pneumoniae* which provides a means of invading human host cells in the presence of plasminogen activator such as tPA or uPA, resulting in infectious diseases, including encephalitis, caused by this bacterium. An understanding of this interaction at a structure level leads to a possible key in combating a particular infection at a very early stage. The results of this study may possibly facilitate in the development of new therapeutic agent such as antibiotics. For example, the information summarized here may be exploited to develop the specific drug target for the enzymes encoded in the genome and/or identification of important epitopes of antigenic protein for drug development.