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# Cyclosporine A and FK506 as Potent Inhibitors of Streptococcus intermedius Intermedilysin-Induced NFAT-I Activation

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#### **Abstract**

Cyclosporine A (CsA) and tacrolimus (FK506), a member of calcineurin inhibitors, inhibit inflammation process as part of immune response. Nuclear activated T cells subfamily NFATI is a trascription factor responsible for the regulation of immune response genes. Streptococcus intermedius, an oral commensal bacterium, has been shown to strongly associate with liver abscess. The S. intermedius strains produce intermedilysin (ILY), which is responsible for the bacterial virulence. Cyclosporine A and FK506 have been widely used to control NFAT activation in most of cell types, however the ability of CsA and FK506 to inhibit ILY-induced NFATI activation remains to be investigated. The aim of this study was to investigate the effect of CsA and FK506 on NFATI activation caused by ILY. Human cholangiocellular cell line HuCCTI was stimulated with various concentrations of ILY. The cell and nuclear morphological change was observed by microscopy analysis. The NFATI nuclear translocation that indicates its activation was detected by immunocytochemistry. The inhibitory effect of CsA and FK506 was tested after 30 min application before ILY treatment by using immunofluorescence microscope. The results showed cell and nuclear shrinkage in ILY-treated cells. The NFATI was translocated to the nuclei in HuCCTI cells, and observed in dose dependent manner. Cyclosporine A and FK506 inhibited ILY-induced NFAT1 nuclear translocation. In conclusion, CsA and FK506 may act as potent inflammation control agents in S. intermedius ILY-infected cells.

Key words: Cyclosporine A, FK506, NFATI, intermedilysin

# **INTRODUCTION**

The transcription nuclear factor of activated T cell (NFAT) which is responsible for immune and inflammatory responses contains five members, NFAT1, NFAT2, NFAT3, NFAT4, and NFAT5. Among them, NFAT1, NFAT2, NFAT3, and NFAT4 are regulated by calcium-calcineurin signalling pathway (Macian, 2005). The signalling pathway regulated by NFAT covers a board number of functions in vertebrate development such as immune system, nervous system, heart and sceletal

muscle, vascular system, lung, bone and skeleton, endocrine system, and skin epithelia (Wu *et al.*, 2007).

Nuclear factor of activated T cell is responsible for the cellular responses against bacterial toxin. A virulence factor produced by *Helicobacter pylori*, CagA, activates NFAT2 in human gastric epithelial cells under regulation of PLC $\gamma$ -Ca<sup>2+</sup>-calcineurin pathway (Yokoyama *et al.*, 2005).



\*Corresponding author e-mail: henisusilowati@yahoo.com That study demonstrates that NFAT2 is a common cellular target of *H. pylori* virulence factors, that might play important role in the pathogenesis of infection by this bacterium (Yokoyama *et al.*, 2005).

Streptococcus intermedius is a normal microflora that is clinically distributed in the oral cavity, gastrointestinal and genitourinary tracts (Whiley et al., 1990; Whiley et al., 1992). This species has related to some deep site infections in brain, liver, and central nervous system (Whiley et al., 1990; Whiley et al., 1992; Yamamoto et al., 1999; Wagner et al., 2006). Some strains of S. intermedius produces intermedilysin (ILY) which acts as critical virulence factor of the infection by this bacterium. Intermedilysin was firts identified and then purified from S. intermedius isolated from liver abscess (Nagamune et al., 1996; Nagamune et al., 2000). Intermedilysin is known to be potential to induce increasing intracellular calcium [(Ca<sup>2+</sup>)i] in polymorphonuclear cells (Macey et al., 2001).

Nuclear factor of activated T cell is very sensitive to the rise in (Ca<sup>2+</sup>)i (Dolmetsch *et al.*, 1998). Prior to its activation, NFAT should be dephosphorylated by a Ca<sup>2+</sup>-dependent enzyme, calcineurin, which facilitates its nuclear translocation (Luo *et al.*, 1996, Jain *et al.*, 1993). Because of the critical role of NFAT activation in generating inflammatory and immune responses, it needs a control mechanism; however, it remains to be investigated. This study subjected to find out the way to control NFAT activation in human cholangiocellular carcinoma cells HuCCT1 by using calcineurin inhibitors cyclosporine A (CsA) and tacrolimus (FK506).

# **METHODS**

# **Cell culture**

Human cholangiocellular carcinoma cell line HuCCT1 was purcased from Japanese Health Science Research Resources Bank (Osaka, Japan). The cells were cultured in RPMI 1640 (Sigma-Aldrich, St Louis, MO, USA) suplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100  $\Box$ g/ml streptomycin at 37°C in the presence of 5% CO<sub>2</sub>.

# Analysis of cell and nuclear morphology

HuCCT1 cells were grown on 15 mm diameter cover slips and were cultured for overnight. Intermedilysin (40 ng/ml) was added to the cells

followed by 24 h incubation. In order to observe the cell and nuclear morphological change caused by ILY we analized ILY-treated cells using phasecontrast and fluorescence microscope (BX-50, Olympus Optical Co. Ltd, Tokyo, Japan).

# **Immunocytochemistry**

In separated experiment, the cells were exposed to various dose of ILY (0 ng/ml, 5 ng/ml, 20 ng/ml, 40 ng/ml) within five min. To invesitigate whether calcineurin inhibitor is able to blok ILYinduced NFAT1 nuclear translocation, CsA (Tocris Cookson, Ellisville, MO, USA) and FK506 (Cayman Chemical, Ann Arbor, MI, USA) were applied for 30 min before ILY treatment. Following stimulation, the cells were fixed in 10% formalin prior to permeabilization using methanol. After blocking with 4% BSA, the cells were exposed to mouse anti-NFAT1 antibody (BD Transduction Laboratories, Lexington, KY, USA) for 45 min at room temperature. Alexa Fluor 488 anti-mouse antibody (Invitrogen, Carlsbad, CA, USA) as secondary antibody was applied for 40 min and followed by nuclear staining with 10 µg/ml of Hoechst 33342. After serial PBS washing, the cells were then examined under a fluorescence microscope.

# **RESULTS**

# Cell and nuclear morphological change

Under light microscope observation, untreated cells showed normal cell morphology (Fig. 1A) with an intact and normal size nuclei (Fig. 1B). Cell morphological change was observed in cells treated with 40 ng/ml ILY (Fig. 1C). These cells showed small rounded apearance with lost of cytoplasmic processus. In addition, these treated cells also demonstrated condensed nuclei under Hoechst 33342 nuclear staining (Fig. 1D).

# Nuclear translocation of NFATI in ILY-treated HuCCTI cells

To determine that ILY could induce activation of NFAT and to investigate its cellular localization in HuCCT1 cell, we performed immunocytochemistry. The results of immunocytochemical analysis in Figure 2A demonstrated that ILY treatment induced nuclear translocation of NFAT1 indicating its activation under ILY stimulation. In the Figure 2B, the results showed increase percentage of NFAT1 positive-



translocation cells in HuCCT1 treated with various dose of ILY.

This observation demonstrated ILY-induced NFAT1 activation in dose dependent manner.

# Inhibitory effect of CsA and FK506 on NFAT1 nuclear translocation

To study the molecular signalling of NFAT1 activation induced by ILY, we did calcineurin inhibitor pretreatment on ILY-exposed HuCCT1 cells. The results demonstrated that calcineurin inhibitors CsA as well as FK506 completely bloked activation of NFAT1 (Fig. 3). The inhibitory effect of CsA and FK506 was detected at the concentration of 100 nM and 5 ng/ml, respectively.

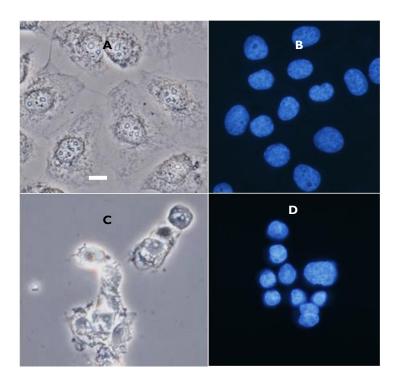
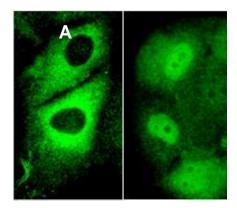


Figure I. Cell morphological change caused by ILY-treatment was observed after 24 h ILY (40 ng/ml) treatment to HuCCTI cells. The untreated cells showed intack cell morphology (A) with smooth nuclear membrane (B). The ILY-treated cells showed smaller cell size with membrane blebbing (C). The nucleic acid staining using Hoechst 33342 revealed smooth rounded nuclei in normal cells (C) and pyknotic nuclei in treated cells (D). Bar indicates 10 μm.





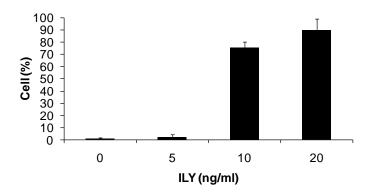


Figure 2. ILY induced nuclear translocation of NFAT1 in HuCCT1 cells. As shown as green fluorescence, the NFAT1 was located in the cytoplasma of untreated cells (A), and translocated to the nuclei of cells treated with 10 ng/ml ILY (B). The percentage of NFAT1-positive translocation cells was increased in line with the increasing ILY concentration. Presented data was the mean ± SD of representative experiments.

# **DISCUSSION**

This study notes that ILY induced cell death in HuCCT1 cells as demonstrated by destructive change of cell morphology and irreversible pyknosis nuclear. This effect was detected in the utilization of nanomolar concentration of ILY, indicating that ILY is strong cytolytic toxin which has capability to induce cell death in HuCCT1 cells. Our result is in line with the previous study reporting the cytotoxic effect of ILY from bacterial culture supernatant on hepatoma HepG2 cells (Sukeno et al., 2005). In clinical sites, the infection caused by bacteria usually raised from a small amount of toxin. Low concentration of toxin in fact is capable to induce Ca<sup>2+</sup> oscillations (Soderblom et al., 2002). This phenomenon was observed in cells treated with sublytic dose of \( \subseteq -hemolysin, cytolysin produced \) by Escherichia coli (Söderblom et al., 2002; Ludwig and Goebel, 2000). These evidences led us to study the early response of cells stimulated with low concentration of ILY, as the initial step to clarify the molecular backgorund of infection caused by *S. intermedius*.

Upon stimulation, NFAT dephosphorylation occurs in short time before its translocation to the cell nucleus to facilitate its binding to a specific binding site in DNA (Shaw *et al.*, 1995). In this study we demonstrated that ILY may induced NFAT1 dephosphorylation followed by nuclear translocation in HuCCT1 cells. This evidence was occure in five min after ILY exposure. This results is supported by previous studies that NFAT dephosphorylation is observed within 5-10 min after stimulation of calcium ionophore (Shaw *et al.*, 1995; Wesselborg *et al.*, 1996; Ruff and Leach, 1995).



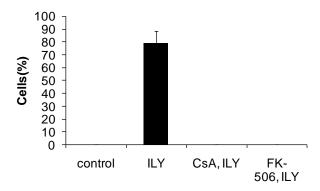


Figure 3. ILY-induced NFATI activation was prevented by calcineurin inhibitor. Pretreatment of 100 nM CsA and 5 ng/ml FK506 completely inhibited the nuclear translocation of NFATI in HuCCTI cells. Data represent the mean ± SD of representative experiments.

NFAT is a molecular target of calcineurin inhibitors (Luo et al., 1996). Pretreatment of CsA and FK506 prevents the activation of calcineurin, subsequently bloks NFAT dephosphorylation and nuclear translocation (Ruff and Leach, 1995; McCaffrey et al., 1993). We demonstrate that CsA and FK506 completely blocked activation of NFAT1. Our study revealed that FK506 was more effective than CsA in preventing ILY-induced NFAT1 translocation. We estimate that the inhibition effect of FK506 was 16-fold more potent than CsA. It might be explained by the evidence that FK506 also affects the CsA-insensitive activation pathway (Almawi and Melemedjian, 2000). The inhibitory effect of FK506 on NFAT activation pathway is initiated by its binding to FK506 binding protein FKBP. This inhibitor binds to FKBP to form FK506-FKBP stable complex (Liu et al., 1991; Addy et al., 2007).

Concidering the ability of CsA and FK506 to prevent NFAT1 activation in ILY-treated HuCCT1 cells, we suggest that these calcineurin inhibitors could function as a potent regulator of inflammatory response in the infection caused by ILY-producer *S. intermedius*. This study may be provide molecular background for the management of inflammation in human biliary disease that is generated by *S. intermedius* infection.

# **CONCLUSION**

In conclusion, CsA and FK506 may act as potent inflammation control agents in *S. intermedius* ILY-infected cells.

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#### **REFERENCES**

Addy, N.A., Fornasiero, E.F., Stevens, T.R., Taylor, J.R. and Picciotto, M.R., 2007, Role of calcineurin in nicotine-mediated locomotor sensitization, *J. Neurosci.* 27, 8571-8580.

Almawi, W.Y. and Melemedjian, O.K., 2000, Clinical and mechanistic differences between FK506 (tacrolimus) and cyclosporine A, Nephrol. Dial. Transplant, 15(2000), 1916-1918.

Dolmetsch, R.E., Lewis, R.S., Goodnow, C.C. and Healy. J.I., 1997, Differential activation of transcription factors induced by Ca<sup>2+</sup> response amplitude and duration, *Nature*, **386**, 855-858.

Jain, J., McCaffrey, P.G., Miner, Z., Kerppola, T.K., Lambert, J.N., Verdine, G.L., Curran, T. and Rao, A., 1993, The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun, *Nature*, **365**, 352-355.

Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I. and Schreiber, S.L., 1991, Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes, Cell, 66(4), 807-815.



- Ludwig, A. and Goebel, W., 2000, Dangerous signals from E. coli toxin, Nat. Med., 6(2000), 741-742.
- Luo, C., Shaw, K.T.Y., Raghavan, A., Aramburu, J., Garcia-Cozar, F., Perrino, B.A., Hogan, P.G. and Rao, A., 1996, Interaction of calcineurin with a domain of the transcription factor NFAT1 that controls nuclear import, *Proc. Natl. Acad. Sci. USA*, **93**, 8907-8912.
- Macey, M.G., Whiley, R.A., Miller, L. and Nagamune, H., 2001, Effect on polymorphonuclear cell function of a human-specific cytotoxin, intermedilysin, expressed by *Streptococcus intermedius*, *Infect. Immun*, **69**, 6102-6109.
- Macian, F., 2005, NFAT proteins: key regulators of T-cell development and function, *Nat. Rev. Immunol.*, **5**, 472-484.
- Nagamune, H., Whiley, R.A., Goto, T., Inai, Y., Maeda, T., Hardie, J.M. and Kourai, H., 2000, Distribution of the intermedilysin gene among the anginosus group streptococci and correlation between intermedilysin production and deep-seated infection with Streptococcus intermedius, J. Clin. Microbiol, 38, 220-226.
- Nagamune, H., Ohnishi, C., Katsuura, A., Fushitani, K., Whiley, R.A., Tsuji, A. and Matsuda, Y., 1996, Intermedilysin, a novel cytotoxin specific for human cells, secreted by Streptococcus intermedius UNS46 isolated from a human liver abscess, Infect. Immun., 64, 3093-3100.
- Ruff, V.A. and Leach, K.L., 1995, Direct demonstration of NFATp dephosphorylation and nuclear localization in activated HT-2 cells using a specific NFATp polyclonal antibody, *J. Biol. Chem.*, **270**, 22602-22607.
- Shaw, K.T.Y., Ho, A.M., Raghavan, A., Kim, J., Jain, J., Park, J., Sharma, S., Rao, A. and Hogan, P.G., 1995, Immunosuppressive drugs prevent a rapid dephosphorylation of transcription factor NFATI in stimulated immune cells, *Proc. Natl. Acad. Sci. USA*, **92**, 11205-11209.
- Söderblom, T., Laestadius, A., Oxhamre, C., Aperia, A. and Richter-Dahlfors, A., 2002, Toxin-induced calcium oscillations: a novel strategy to affect gene regulation in target cells, *Int. J.*

- Med. Microbiol., **291**, 511-515.
- Sukeno, A, H. Nagamune, R.A. Whiley, S.I. Jafar, J. Aduse-Opoku, K. Ohkura, T. Maeda, K. Hirota, Y. Miyake, H. Kourai. 2005. Intermedilysin is essential for the invasion of hepatoma HepG2 cells by Streptococcus intermedius. Microbiol. Immunol. 49, 681-694.
- Wagner, K.W., Schön, R., Schumacher, M., Schmelzeisen, R. and Schulze, D., 2006, Case report: brain and liver abscesses caused by oral infection with Streptococcus intermedius. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod., 102, e21-e23.
- Wesselborg, S., Fruman, D.A., Sagoo, J.K., Bierer, B.E. and Burakoff, S.J., 1996, Identification of a physical interaction between calcineurin and nuclear factor of activated T cells (NFATp), J. Biol. Chem., 271, 1274-1277.
- Whiley, R.A., Beighton, D., Winstanley, T.G., Fraser, H.Y. and Hardie, J.M., 1992, Streptococcus intermedius, Streptococcus constellatus, and Streptococcus anginosus (the Streptococcus milleri group): association with different body sites and clinical infections, J. Clin. Microbiol., 30, 243-244.
- Whiley, R.A., Fraser, H., Hardie, J.M., Beighton, D., 1990, Phenotypic differentiation of Streptococcus intermedius, Streptococcus constellatus, and Streptococcus anginosus strains within the "Streptococcus milleri group", J. Clin. Microbiol., 28, 1497-1501.
- Wu, H., Peisley, A., Graef, I.A., Crabtree, G.R., 2007, NFAT signaling and the invention of vertebrates. *Trends Cell Biol.* 17, 251-260.
- Yamamoto, M., Fukushima, T., Ohshiro, S., Go, Y., Tsugu, H., Kono, K. and Tomonaga, M., 1999, Brain abscess caused by *Streptococcus intermedius*: two case reports, *Surg. Neurol.*, **51**, 219-222.
- Yokoyama, K., Higashi, H., Ishikawa, S., Fujii, Y., Kondo, S., Kato, H., Azuma, T., Wada, A., Hirayama, T., Aburatani, H. and Hatakeyama, M., 2005, Functional antagonism between *Helicobacter pilory* CagA and vacuolating toxin VacA in control of the NFAT signaling pathway in gastric epithelial cells, *Proc. Natl. Acad. Sci. USA*, **102**, 9661-9666.