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Scientific Basis for Traditional Medicines in Renal Disease

Ching-Yuang Lin*

Children's Hospital, Changhua Christian Hospital, Taiwan;
Institute of Medical Research, College of Health Sciences,
Chang Jung Christian University, Taiwan

Abstract: Cordyceps Sinensis (CS) is a parasitic fungus that has been used as a Chinese medicine for a long time in the treatment of nephritis. In this study, we established an *in vitro* model that showed that cultured human mesangial cells (HMC) stimulated with interleukin-1 (IL-1) plus IL-6 can cause mesangial cell proliferation, increasing production of chemical mediators and superoxide anion. An *in vivo* model also proved that this culture medium might lead to renal injury with hematuria and proteinuria. We cultured HMC, and then an HMC activating model with HMC incubated with IL-1 and IL-6 was established. We fractionated the crude methanolic extracts from fruiting bodies of CS with the use of this *in vitro* inhibition of HMC activation model as our assay method. Then we established an IgAN animal model with R36A (Pneumococcal C-polysaccharide purified from Streptococcus pneumoniae) as antigen and anti-R36A IgA monoclonal antibody to form nephritogenic IgA-IC, which can induce hematuria and proteinuria in mice with IgA deposition in the mesangial area. The fruiting bodies were extracted by silica gel column chromatography. One out of 6 column fractions, F-2, significantly inhibited the HMC activation by IL-1 plus IL-6. The acute toxicity test with male institute of Cancer Research mice showed no liver toxicity or mutagenicity. The mice in the IgAN model fed with 1% F-2 in diet had significant reduction of hematuria and proteinuria together with histopathologic improvement. Therefore this fraction was then purified by silica gel column chromatography and high-performance liquid chromatography, which yielded a purified compound H1-A, which can suppress the activated HMC and alleviate IgAN (Berger's disease) with clinical and histologic improvement. These results give us a new regimen for the treatment of patients with IgAN in the future.

Keywords: Cordyceps Sinensis (CS); human mesangial cells (HMC); IgA Nephropathy (Berger's disease); H1-A; Active fraction.

1. Introduction

Cordyceps sinensis (CS), one of the well-known traditional Chinese medicines, is a fungus that develops stroma and is found on the larvae of the Lepidoptera caterpillar [1-3]. Recent studies have demonstrated its multiple pharmacologic actions such as reducing damage to renal tubules and protecting the Na^+, K^+-ATPase on cellular membranes, an action that is associated with a reduction in cellular lipid peroxidation [4]. It can decrease chronic renal insufficiency [5]. In rats, it alleviated hematuria and reduced the elevation of serum creatinine [5]. An extract of CS increased the volume of blood flow in

* Corresponding author; e-mail: 100966@cch.org.tw
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the coronary arteries of animals under experiments [6]. It also reduced resistance and pressure in the arteries, brain, and peripheral vascular system [6]. CS also promotes platelet formation [7]; helped in prevention of hypoxia, and acted as a monoamine oxidase inhibitor [8]. However, no purified compounds from CS have been used before to evaluate the previously mentioned pharmacologic actions. None of the research conducted on alleviating the histologic lesions of renal disease was based on any animal model of IgA mesangial nephropathy (IgAN).

IgAN is a chronic disease [9]. Renal function is usually normal at its onset, but gradually a progressive decline in glomerular filtration rate occurs in some cases. Extensive long-term follow-up studies in France, Italy, Spain, and Taiwan have indicated that progressive renal insufficiency develops in 20% to 30% of patients with IgAN 20 years or more after the disease is initially discovered [10, 11]. Unfortunately, at present no therapeutic maneuvers have been proved to be consistently effective. Hence, there is a pressing need for development of a curative substance. In this regard, earlier we have studied the pathogenesis of IgAN and have hypothesized that mesangial deposition of nephritogenic IgAIC in glomeruli may activate resting mesangial cells [12]. The activated mesangial cells start to release cytokines including IL-1, IL-6, and tumor necrosis factor-α. These cytokines cause mesangial cells to release growth factors are similar to those of the autocrine system, in which a vicious cycle of mesangial proliferation is induced with the release of chemical mediators, such as platelet-derived growth factor and transforming growth factor-β, in addition to IL-1 and IL-6 [12]. The actions of these cytokines and growth factors are similar to those of the autocrine system, in which a vicious cycle of to mesangial proliferation is induced with release of cemical mediators such as platelet activation factor, prostaglandin E_2, thromboxane B_2, neutral proteinase, and free radicals including superoxide anion, which all lead to the glomerular injury and scloerosis [12]. However, nephritogenic IgAIC in humans is still unknown. To solve this problem first, we established an *in vitro* model that cultured HMCs stimulated with IL-1 plus IL-6, which can cause HMCs proliferation, increased production of chemical mediators and superoxide anion [13].

The first study was designed to investigate this possibility with regard to compounds from Chinese medicinal herbs. Fifteen species of Chinese herbs were collected and extracted with MeOH. The herbs were: *Eclipta prostrata, Selaginella tamariscina, Springlxeris chinensis, Poygonum hypoleucum Ohwi, Glechorna hederacea, Scutellaris rivularis, Diconodra micrenta, Centella asiatica, Verbena officinalis, Abrus cantonensis, Agrinomia pilosa, Condonacanthus paucifiorus, Xanthium strumarium, Daemonoropus margaritae, and cordycep smensis*. The effects of methanolic crude extracts of these herbs on proliferation and cytokine gene expression and production in human mesangial cells were determined.

CS crude active extract was the most potent screened Chinese herbs which inhibited human mesangial cell proliferation activated by interleukin-1β and interldukin-6. CS crude active extract also decreased interldukin-1β and tumor necrosis factor-α production. Moreover, TNF-α mRNA expression was inhibited by CS crude active extract. It is unlikely that cytotoxicity was involved, because no cell death was observed. We hypothesize that the inhibitory mechanism of these Chinese herbs may be related to the impairment of gene expression and production of cytokines in human mesangial cells. The next step was planned for the isolation of pure compounds from CS crude active extract and the elucidation of their mechanism of action.

Before we start to isolate the pure compound, we need animal model. The requirements for animal models include both

specificity and the capacity for developing renal histopathologic lesions that are similar to those found in the corresponding human disorders. In view of the previously described considerations, the IgAN models developed by Rifai [14] were adopted for the experiments used in developing this investigation. Therefore in this study, the selected antigen was R36A, a purified C-poly-saccharide obtained from the cell wall of *Streptococcus pneumoniae* [15], and the antibody used was the IgA monoclonal antibody that is specific to R36A to form nephritogenic IgAIC [14, 16], which can induce hematuria and proteinuria in mice with IgA deposition in the mesangial area [17].

A total of 6 subfractions were obtained from crude methanolic extract of CS fruiting bodies. When activated HMCs by IL-1 and IL-6 were used as target cells, F-2 had the strongest inhibitory effect on the proliferation of activated HMCs. Thus, we could observe the dose-responsive inhibitory action on activated HMCs by F-2.

In this IgAN mouse model, renal tissue stained by hematoxylin-eosin stain revealed histolgoic changes with mesangial cells proliferation and mesangium expansion similar to those found in human IgAN. The immuno-fluorescent stain also revealed mesangial depositions of IgAN and C3 similar to those found in human IgAN. On clinical evaluation, these mice were found to have hematuria and proteinuria. The histopathologic improvement of IgAN in mice fed a normal diet containing F-2. There was a significant decrease in mesangial cell proliferation and mesangial expansion in mice fed with a normal diet containing 1% F-2. The immuno-fluorescent intensity of IgA and C3 on the mesangial area was also significantly decreased in mice fed a normal diet containing 1% F-2. On clinical evaluation hematuria, proteinuria, or both (urinary albumin/Cr, 0.22 ± 0.33 in 1% F-2-treated group vs 0.38 ± 0.04 in untreated group, $P<0.01$) were also significantly decreased on day 7 after mice were fed a normal diet containing 1% F-2.

Then asult toxicity test was performed. No statistical difference was seen in body weight and liver function including ALT, AST, and cholesterol level except an increase in the ratio of liver weight over body weight (normal vs 2% F-2: 5.44 ± 0.17 vs 6.71 ± 0.13, $P<0.01$). Significantly increased liver cytochrome –450 content was seen in the F-2-treated group (normal vs 2% F-2: 0.78 ± 0.08 vs 1.43 ± 0.09, $P<0.01$). These results show that F-2 could both suppress proliferation of activated HMCs *in vitro* and prevent the exacerbation or even cause improvement of IgAN in an animal model *in vivo*. F-2 also had low toxicity. For these reasons we started to identify the effective compound in CS.

To extract the pure active compound from the F-2, we used the entrie isolation process in each chromatographic cycle and finally by high-performance liquid chromatography from methanol extraction to final purification of compound H1-A. With the use of the previously described *in vitro* procedures, the strongest activity compound H1-A was obtained in its pure form. The structure of compound H1-A was obtained after the nuclear magnetic resonance spectrum and Mass study. The molecular formula of H1-A was $C_{28}H_{42}O_2$. To confirm the potential applicability of H1-A and to check for obvious toxicity or mutagenicity, Ames test and acute toxicity test were conducted on mice from the Institute of Cancer Research and the results were found similar to the F-2. They demonstrated either no acute toxicity or mutagenicity. *In vitro* inhibition on HMCs by H1-A, the GI_{50} (Growth inhibition$_{50}$) was 40 μmol/L.

Simultaneously adding either F-2 or H1-A on the HMCs stimulated by IL-1 plus IL-6 showed that fluorescence intensity of HMCs in the S and G0 + G phase of the cell cycle was similar to that of unstimulated HMCs. Despite the previously mentioned inhibitory effect on cell proliferation, neither F-2 nor

H1-A affected cell viability, nor did they exert cytotoxic phenomena, as judged by trypan blue incorporation, lactate dehydrogenase release from cells after 72 hours incubation, and MTT assay.

Apoptosis was observed in cultured human mesangial cells after incubation with H1-A at concentrations of 12.5 and 25 μ mol/L. There was no apoptosis observed in cells treated with IL-1β/PDGF-BB only.

The addition of IL-1β and PDGF-BB to cultured human mesangial monolayers resulted in a time-dependent increase in tyrosine phosphorylation of several proteins. The induction was visible at a minimum of 5 minutes and a maximum of 30 minutes. We noted that several proteins with various molecular masses were phosphorylated after incubation with IL-1β and PDGF-BB. Three proteins (with apparent molecular masses of 20-30, 60-70, and 200-300 kD) were phosphorylated after incubation with IL-β and PDGF-BB, and the phosphorylation was inhibited by the addition of H1-A in concentrations of 6.25, 12.5, and 25 μmol/L, consistent with a dose-dependent response. These data demonstrate that IL-1β and PDGF-BB stimulated protein tyrosine phosphorylation in human mesangial cells, a reaction that was inhibited by H1-A.

To determine whether IL-1β and PDGF-BB induced the tyrosine phosphorylation of Bcl-2 or Bcl-XL, we immunoprecipitated whole-cell lysates with Bcl-2 or Bcl-XL, then conducted immunoblotiing with an antibody specific for Bcl-2 or Bcl-XL. Proteins with a molecular mass of 20 to 30 kD were found to be Bcl-2 or Bcl-XL, both with a molecular mass of 26 kD. Then we probed the same nitrocellulose papers with an antibody specific for tyrosine phospho-proteins, after stripping tem off, to confirm the tyrosine phosphorylation of Bcl-2 or Bcl-XL. Bcl-2 or Bcl-XL was detectable in lysates from control or IL-1β/PDGF-BB-activated mesangial cells. The addition of H1-A in various concentrations did not change the intensity of expression. The tyrosine posphorylation of Bcl-2 was induced with IL-1β/ PDGF-BB and inhibited by H1-A in concentrations of 12.5 and 25 μmol/L in a dose-dependent manner. The tyrosine phosphorylation of Bcl-XL was induced with IL-1β/PDGF-BB and inhibited by H1-A in concentrations of 6.25, 12.5, and 25 μmol/L. In addition, we applied anti-MAP4, anti-Sre, and anti-Zo-1 to identify other tyrosine-phosphorylated proteins; however, these attempts were unsuccessful (data not shown). These observations demonstrate that the tyrosine phosphorylation of Bcl-2 or Bcl-XL in human mesangial cells can be inhibited by H1-A.

The cordyceps grows the fungi in -form a worm. Cordyceps is considered to be a pharmaceutical kidney tonic in China over the past 2000 years. In recent studies, the cordyceps fraction is considered to have potential protecting the liver [18], in improvement of the immunity [19]; in protection of cardiovascular, endocrine and kidney disease [20]. There is only limited clinical testing in renal disease to use cordyceps. Thirty patients with chronic kidney failure (CRF) in the course of a research [21], who used cordyceps found creatinine clearance rate and reduced blood urea nitrogen (BUN) and serum creatinine (SCr). The combined treatment of Cordyceps and Jin Shui Bao to a chronic kidney failure patient, showed decreased serum BUN and Cr levels, and reduction in the urine protein and blood pressure. In the clinical treatment of 61 systemic lupus erythematus (SEL) with nepritis patients without corticosterone and cyclophospamide showed that after intake of 2-4 grams of cordyceps powder and 0.6 grams of artemisinin for 3 years, prevented renal function deterioration and decreased proteinuria. Also, cordyceps has therapeutic efficacy in the treatment of iatrogenic nephrotoxicity [22]. It has been demonstrated that CS can reduce cyclosporin induced nephrotoxicity. Sixty nine patients with renal transplants

received cyclosporine as immunosuppressive regimens with cyclosporine induced nephrotoxicity were divided randomly divided into two groups. Control group received cyclopsorin only and the another group received cyclosporin and cordyceps (3g/per day). The results showed that the cordyceps group had decreased serum BUN and Cr levels. In an another report, mouse treated with cyclosporine-A and cordyceps significantly reduced interstitial edema tubular necrosis and fibrosis compared to the group which received only cyclosporin A [23]. The preventive mechanism of iatrogenic nephrotoxicity by CS was considered to be the effect of antioxidant [24]. Because the mouse of reperfusion supports the vitamin E has advantage of showing to the kidney [25]. Also, Shahed et al. (2001) showed that water fraction of CS had beneficial effect on the kidney in a ischaemia/reperfusion mice model. CS water fraction could reduce serum Cr level, and the MCP-1 and TNF-a gene expression was also suppressed [26].

In this study, first, we relied on our previous study, in which IL-1 plus IL-6 stimulated HMC to proliferate with concomitant morphologic change *in vitro* [12]. The activated HMC produced PAF and superoxide anion. With this method, we established an *in vitro* assay system. We also demonstrated an IgAN animal model. With these *in vitro* and *in vivo* assay systems, the proliferation inhibitory activities of fraction F-2, C-11, T-4, and pure compound H1-A from methanolic extracts CS fruiting bodies were isolated. H_1A is an ergosterol. The molecular formula is $C_{28}H_{42}O_2$ and molecular weight is 410. There is no corticosteroid effect (data not shown).

H1-A decreased the population of cells in S-phase and increased those in the $G_0 + G_1$ phase. With the use of TUNEL immunoflu-orescence staining, we showed that H1-A conocentrations of 12.5 and 25 μ mol/L can cause apoptosis in cultured uman mesangial cells. We also demonstrated that IL-1β and PDGF-BB can enhance protein tyrosine phosphorylation in human mesangial cells and that this activation can be inhibited by H1-A. Bcl-2 and Bcl-XL are two proteins that we identified with the use of immunoprecipitation and immunoblotting.

We demonstrated that the beneficial effects observed under *in vitro* result from the effect of F-2 on inhibition of proliferation in activated HMCs. *In vivo* experiment, we observed decreasing mesangial cell proliferation in animals with IgAN, while treated with F-2. However, treated animals also had a marked reduction in immune complex deposition. Therefore, other potential effects of F-2 that might account for the reduction in disease in treated animals should be explored for reduction in renal blood flow leading to reduced immune complex deposition, complement inhibitory effects, and alteration in function of the mononuclear phagocyte system. The H1-A blood level in the mice fed with F-2 per os was detected with high-performance liquid chromatography. Since, pharmacokinetic and hemodynamic studies have not been done, hence, we have no information on the effect of either F-2 or H1-A on the renal blood flow. From the preliminary data in our laboratory, we know that both H1-A and F-2 are without complement inhibitory effects (data not shown). Thus, the most possible mechanism in the reducing immune-complex deposition may be through the alteration in function of the mononuclear phagocyte system.

Several immunosuppressive and cytotoxic drugs have been used to treat glomerulonephritis. These include glucocorticoids, cyclophosphamide, cyclosporine and FK-506 [27, 29]. Some of these drugs have systemic effects, and others have direct effects on renal cells [30]. Their mechanism and sub-cellular regulation are not well known. In hematopoietic cells, glucocorticoids activate NF-κB and induce apoptosis [31]. FK-506 and cyclosporine drugs inhibit calcineurin, nuclear translocation of NF-AT and also interfere with calcineurin interaction with

phospho-NF-AT [32]. However, little has been published about their pharmacologic effects in mesangial cells. In this study, we showed that H1-A inhibited mesangial proliferation without causing cytolysis. The progression of the cell cycle was modified, and some of the mesangial cells were induced to develop apoptosis. These effects may be related to some tyrosine phosphoprotein intermediates. Two of them were identified as Bcl-2 and Bcl-XL. However, we did not observe a change in the expression of these two antiapoptotic members. The implications of these results in human renal disease or lupus nephritis remain to be clarified.

In conclusion, H1-A may modulate the effects of Il-1β/PDGF-BB on glomerular mesangial cells, and Bcl-2/Bcl-XL may be a mediator at the molecular level. Because the sub-cellular effect of H1-A is different from that of glucocorticosteroids, cyclosporine, or FK506, it may be an alternative to treat human glomer-ulonephritis or may be used as adjunctive therapy. Additionally, this report suggests that tyrosine kinase, Bcl-2, and Bcl-XL are potential targets for pharmacologic interventions in human renal disease.

References

[1] Kobayasi, Y. 1941. The genus Cordyceps and its allies. Science Report Tokyo Bunrika Daigaku, 5: 253-60.

[2] McEwen, F.-L. 1963. Insect pathology 2, New York: Academic Press; pp. 273-290.

[3] Simitsu, D. 1978. Cordyceps, Green Books 51. Tokyo, Japan: New Science Co.

[4] Tlan, J., Chen, X.-M. and Li, L.-S. 1991. Effects of Cordyceps sinensis, rhubarb and serum renotropain on tubular epithelial cell growth. Chung-Hsi-I-Chieh-Ho-Tsa-Cin., 11: 547-9.

[5] Cheng, Q. 1992. Effect of Cordyceps sinensis on cellular immunity in rats with croinci renal insufficiency. Chung-Hsi-I-Chieh-o-Tsa-Chin., 72: 27-29.

[6] Feng, M.-G., Zhou, Q.-G. and Feng, G.-H. 1987. Vasodilating effect of cultured cordyceps sinensis mycelia in anesthetized dogs. Chung-Yao-Tung-Pao., 12: 41-45.

[7] Chen, D.-M. 1987. Platelet hemopoiesis and ultrastructure observations in mice treated with natural cordyceps sinensis and its cultured mycelia. Chung-Yao-Tung-Pao., 12: 47-49.

[8] Wang, Q. and Zhao, Y. 1987. Comparison of some pharmacological effects between Cordyceps and Cepalosporium sinensis Cen sp. Nov. Cung-Yao-Tung-Pao., 12: 42-44.

[9] Lin, C.-Y. 1994. Treatment of IgA nephropathy. Suringer Semin Immunopathol., 16: 121-7.

[10] Jennette, J.-C. 1998. Immunohistology of IgA nephropthay. American Journal of Kidney Diseases, 12: 348.

[11] Julian, B.-A. 1988. First national symposium on IgA nephropathy. American Journal of Kidney Diseases, 10: 337.

[12] Chen, W.-P., Chen, A. and Lin, C.-Y. 1995. *In vitro* effects of interleukins on human mesangial cells: implications for glomeruloenphritis. Journal of Pathology, 175: 327-337.

[13] Chen, A., Chen, W.-P. and Shieu, L.-F. 1994. Pahtogenesis of IgA nephropathy: in vitro activation of human mesangial cells by IgA immune complex leads to cytokine secretion. Journal of Pathology, 173: 119-26.

[14] Rifai, A. 1987. Experimental models for IgA-associated nephritis. Kidney International, 31: 1-7.

[15] Liu, T.-Y. and Gotsclich, E.-C. 1963. The chemical composition of pneumococcal C-polysaccharide. Journal of Biological Chemistry, 238: 1928-1934.

[16] Montinaro, V., Hevey, K. and Aventaggiato, L. 1992. Extrarenal cytokines modulate the glomerular response to IgA immune complexes. Kidney International, 42: 341-353.

[17] Imal, H., Chen, A. and Endoh, M. 1987. Influence of the antigen on experimental IgA nephropathy. Seminars in Nephrology, 7: 283-285.
[18] Liu, Y.-K. and Sen, W. 2003. Inhibitive effect of cordyceps sinensis on experimental hepatic fibrosis and its possible mechanism. World Journal of Gastroenterology, 9: 29-33.
[19] Koh, J.-H, Yu, K.-W., Suh, H.-J., Coi, Y.-M., Ahn, T.-S. 2002. Activation of macrophages and the intestinal immune system by an orally administered decoction from cultured mycelia of Cordyceps sinensis. Bioscience Biotechnology and Biochemistry, 66: 407-411.
[20] Zhu, J.-S., Halpern, G.-M. and Jones, K. 1998. The scientific rediscovery of an ancient Chinese herbal medicine: Cordyceps sinensis: part I. Journal of Alternative Complementary Medicine, 4: 289-303.
[21] Chen, Y.-P., Liu, W.-A., Shen, L.-M. and Xu, S.-N. 1986. Comparisons of fermented Cordyceps mycelia and natural Cordyceps sinesis in treating 30 patients with renal failure. Chin. Tradit. Herbal Drugs, 17: 256-258.
[22] Lu, L. 2002. Study on effect of Cordyceps sinensis and artemisinin in preventing recurrence of lups nepritis. Zhongguo Xi Yi Jie He Za Zhi Zhongguo Zhongxiyi Jiehe Zazhi/Chinese J. Intergrated Traditional Western Med./Zhongguo Zhong Yi Yi Jie He Xue Hui, Zhongguo Zhong Yi Yan Jiu Yuan Zhu Ban., 22: 169-171.
[23] Zhao, X. and Li, L. 1993. Cordyceps sinensis in protection of the kidney from cyclosporine A nephrotoxiciyt. Cung-Hua I Hsueh Tsa Chih., 73 (410-2): 447.
[24] Li, S.P., Zhao, K.-J. and Ji, Z.-N. 2003. A polysaccharide isolated from Cordyceps sinensis, traditional Cinese medicine, protects PC 12 cells against hydrogen peroxide-induced injury. Life Sciences, 73: 2503-2513.
[25] Zurovsky, Y. and Haber, C. 1995. Antioxidants attenuate endotoxin-gentamicin induced acute renal failure in rats. Scandinavian Journal of Urology and Nephrology, 29: 147-154.
[26] Shahed, A.-R., Kim, S.-I. and Shoskes, D.-A. 2001. Down-regulation of apoptotic and inflammatory genes by Cordyceps sinensis extract in rat kidney following iscemia/reperfusion. Transplantation Proceedings, 33: 2986-2987.
[27] Austin, H.-A. and Balow, J.-E. 1999. Natural history and treatment of lupus nephritis. Seminars in Nephrology, 19: 2-11.
[28] Fu, L.-W., Yang, L.-Y., Chen, W.-P., Lin, C.-Y. 1998. Clinical efficacy of cyclosporine and neural treatment of pediatric lupus nephritis with heavy proteinuria. Br. Journal of Rheumatology, 37: 217-221.
[29] Nakahama, H., Obata, K., Sugita, M., Orio, M., Oka, K. and Moriyama, T. 1999. Effect of FK506 in the treatment of autoimmune glomerulonephritis in brown Norway rat. Nephron, 81: 421-427.
[30] Kiberd, B.-A. and Young, I.D. 1994. Modulation of glomerular structure and function in murine lupus nephritis by methylprednisolone and cyclophosphamide. Journal of Laboratory and Clinical Medicine, 124: 496-506.
[31] Sikora, E., Grassilli, E., Radziszewska, E., Bellesia, E., Barbieri, D. and Franceschi, C. 1993. Transciption factions DNA-binding activity in rat thymocytes undergoing apoptosis after heat shock or dexamethasone treatment. Biochemical and Biophysical Research Communications, 197: 709-715.
[32] Wang, H.-C., Pathan, N., Etell, I.M., Krajewski, S., Yamaguchi, Y. and Shibasaki, F. 1999. Ca^{2+} induced apoptosis through calcineurin dephosphorylation of BAD. Science, 284: 339-343.