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The Ganoderma Extracts from Tein-Shan Ganoderma Capsule Suppressed LPS-Induced Nitric Oxide Production in RAW264.7 Cells

Chien-Hua Chu, Shu-Ping Ye, Hui-Chung Wu, Chi-Ming Chiu*
Department of Biotechnology, Ming Chuan University, Gui-Shan 333, Taoyuan, Taiwan, R.O.C

Abstract

Ganoderma lucidum is a high value mushroom used in oriented medicine for its anti-tumor and immunoregulatory effects. Here we determined one commercial *Ganoderma* product, Tein-Shan *Ganoderma* (TSG), for its immunomodulatory activity. In murine monocytic RAW264.7 cell line model, we found that the extracts of TSG did not affect cell viability of macrophages but caused cell morphology change under the treatment of TSG methanol extracts. Moreover, TSG extracts significantly inhibited nitric oxide (NO) synthesis in RAW264.7 cells stimulated by LPS. The suppression of NF-κB activation by LPS is correlated with the reduction of NO production under TSG administration. Our study shows that TSG plays a role in the suppression of inflammatory response in monocytic cells.

Keyword: *Ganoderma lucidum*, immunomodulation, inflammation, nitric oxide, NF-κB
Corresponding author: Chi-Ming Chiu [cmchiu@mail.mcu.edu.tw]
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Introduction

Traditional mushroom *Ganoderma lucidum* (lingzhi) is usually applied in Chinese or Asian medical usage. Numerous studies have demonstrated its effect on anti-tumor, anti-metastasis, immunomodulation, anit-epileptic, even on wound healing effects [1, 2, 3, 4]. There are at least three active components reported from the analysis of *Ganoderma* extracts. The water soluble fractions from *Ganoderma lucidum* contain

specific protein-Ling Zhi-8 (LZ-8) and proteoglycan PS-G. Both are demonstrated with immunomodulatory effect. PS-G is able to induce immune activity to suppress tumor growth [5], while LZ-8 protein can induce ER stress autophagy and p53-dependent growth arrest in gastric and lung cancer cells, respectively [6, 7]. The other one type of *Ganoderma* content is triterpenes extracted from organic solvent(s) such as methanol. Triterpenes of *Ganoderma* are reported with anti-tumor activity [8]. Anti-inflammatory response has also been found by this content [9].

Recent studies of *Ganoderma lucidum* in immunomodulation suggests that the traditional medicine can be used in the therapy of allergic or inflammatory responses. PS-G of *Ganoderma lucidum* can stimulate the proliferation of T cells and the maturation of dendritic cells under the treatment of dust mites' allergen-Der P1, but it suppressed the Th2 inflammatory activity in mouse model and phagocytic activity in dendritic cells [10, 11]. Triterpene extracts of *Ganoderma* has been identified in the reduction of inflammatory cytokines TNF-α, IL-6 and inflammatory mediators like nitric oxide (NO), prostaglandin E2 (PGE_2) in lipopolysaccaride (LPS)-induced murine RAW264.7 monocytic cells through NF-κB and AP-1 pathways [9]. Thus, the *Gonaderma* extracts are involved in the regulation of immune response.

Here we tried to determine the immune-modulatory activity of Tein-Shan *Ganoderma* (TSG) powder. We need to verify whether the product could be applied in the autoimmune therapy. Initially, we examine the TSG extracts in the inflammatory response *in vitro*. Monocytic cell line study may provide us preliminary evidence for future studies in animal model.

Material and Methods

Preparation of TSG extracts

Tein-Shan *Ganoderma* (TSG) capsules are obtained from Lo Kuei Ying Company. The powder 0.05 g was extracted in 2.5 ml distilled water or 80% methanol under sonication for 30 minutes on ice water. Then the mixtures were centrifuged at 4^{o}C, 13,000 rpm for 20 minutes to remove insoluble particles. The supernatants from extractions were concentrated by Speed-Vec. The extracts were freeze at -80^{o}C until the following assay.

Cell culture、treatment and transfection

Mouse monocytic RAW264.7 and human THP-1 cell lines were grown in DMEM and RPMI-1640 medium with 10% fetal bovine serum, respectively, at 37^{o}C, 5% CO_2 incubator.

RAW264.7 cells were plated $5x10^5$ cells per well of 24-well plates. After overnight growth, the cells were treated with 0.25 μg/ml LPS in combination with different dosages of TSG extracts.

THP-1 cells were transiently transfected with NF-κB binding promoter luciferase reporter (pGL4.32[*luc*2P/NF-κB-RE/Hygro]) and constitutive internal control reporter CMV-Renilla (pGL4.75[*hRluc*/CMV]) in combination with CMV-p50 (a gift from Dr. CJ Chang, NTU, Taipei) by PolyJet *in vitro* DNA transfection reagent (SignaGen, MD, USA). The dosage of LPS treatment in THP-1 cells was 1 μg/ml for overnight culture.

Nitric oxide assay

The culture medium from treated cells was mixed with Griess reagent (Sigma-aldrich) in 1:1 volume proportion. Then the relative nitric oxide level was determined by ELISA reader at 540 nm absorbance.

Cell viability assay

Treated cells were washed with PBS (phosphate buffered saline) and then add a tetrazolium compound MTS into culture plates. Cell proliferation activity was determined by CellTiter 96® AQueous One Solution Reagent (Promega, Mannheim, Germany) following the procedures described in technical bulletin.

Luciferase reporter assay

293T cells were cotransfected with pGL4.32 and CMV-Renilla vectors (Promega, Mannheim, Germany). After transfection for 16 hours, the cells were incubated with LPS for more 12 hours before reporter activity determination. The cell lysates were harvested by Dual-Luciferase® Reporter Assay Kit (Promega, Mannheim, Germany). The relative luciferase activity of 2 μg lysate was detected by FB12-single tube luminometer (Berthold Detection System, Pforzheim, Germany).

Statistical analysis

Statistical data were calculated by Microsoft Excel 2010 software. Data are presented as the mean ± standard error of the mean (SEM). The significance was showed using Student's t-test. The symbol # or * indicates $p < 0.05$; ## or ** indicates $p < 0.01$; and *** indicates $p < 0.001$ compared with the control of LPS treatment. Each experimental data consists of three independent replicates.

Results and Discussion

Administration of Tein-Shan *Ganoderma* extracts has no effect on the cell viability of monocytic RAW264.7 cells

Since Tein-Shan *Ganoderma* (TSG) extracts has been demonstrated to show anti-carcinogenic effect [12], certain concentration of water or methanol extracts should have cytotoxic effect on cells. Here we try to differentiate the immune response to macrophage cells from the toxicity to tumor cells. Thus cell viability assay provided us suitable concentration of TSG extracts applied in the cell line-RAW264.7 cells for the following immune assay. Results of MTS assay found that TSG extracts neither from water extraction nor from methanol one affected the cell viability of monocytic cells under the TSG dosages of 25 μg/ml or 100 μg/ml (Figure 1). It suggests that the TSG dosages used in following test cannot cause cell death.

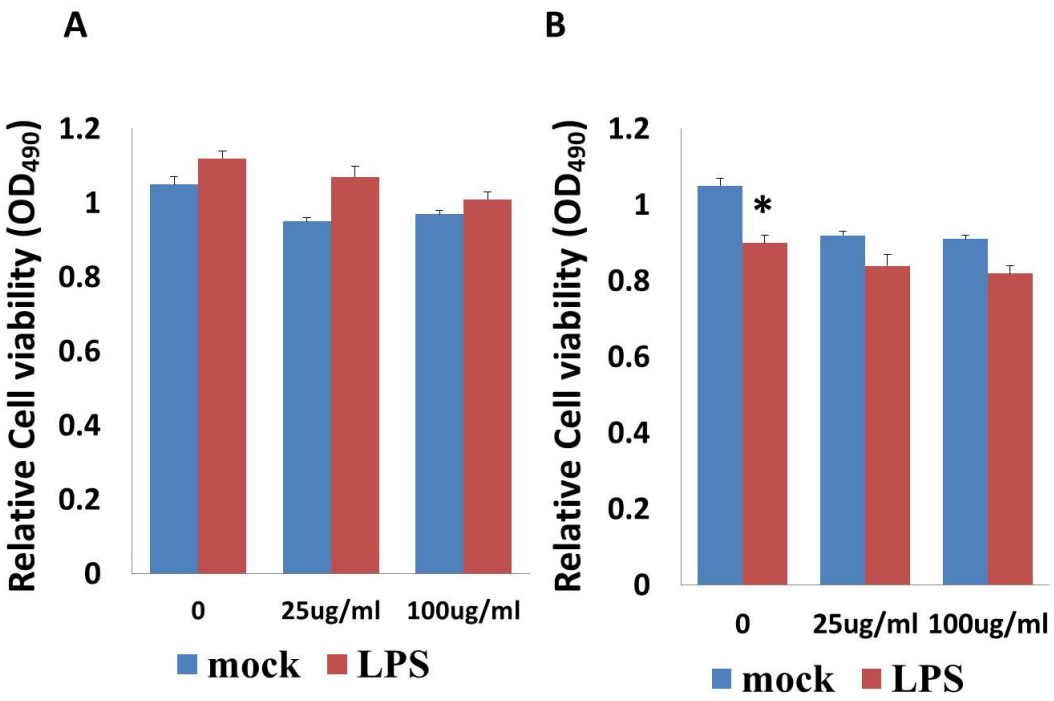

Fig 1. TSG water or methanol extracts did not affected cell viability of RAW264.7 cells. (A) Two concentrations (25 μg/ml or 100 μg/ml of TSG powder weight in methanol solvent) of 80% methanol extracts were applied in the culture medium with 0.25 μg/ml LPS stimulation for 16-18 hours before MTS assay. Blue bar groups mean no LPS treatment controls, whereas red bar groups indicate LPS treatment ones. Student T test values for each treatment compared with no TSG addition control have no significant variations (P value >0.1). (B) Similar to experiments in (A) the treatment was replaced with TSG water extracts. Two dosages (25 μg/ml or 100 μg/ml of TSG powder weight in water) were added into medium with LPS. Blue bar groups indicate as no LPS stimulation controls, whereas red bar groups indicate as LPS treated ones. Student T test values for each treatment compared with no TSG control also have no significant difference except no TSG treatment in the presence of LPS. P value<0.1 was indicated as *.

Methanol extracts of TSG changed cell morphology of RAW264.7 cells

To compare the response of TSG water extracts with the one of methanol extracts, we characterize the cell morphology after 16-18 hours treatment. We intriguingly identified that methanol extracts (100 μg/ml) of TSG caused the extension in cell shape distinct from the round-shape normal morphology in RAW264.7 cells (Fig. 2B, 2D). The difference in cell shape was not found in the shame control treated with identical amount of 80% methanol alone (Fig. 2A). Moreover, the change in cell shape was also not observed in TSG water extracts treatment (Fig.2E, 2F). These results suggest that the organic soluble components like triterpene probably induced the differentiation of monocytes into macrophage-like cells. Nevertheless, the mechanism of cell morphology change on cytoskeleton remained to be determined.

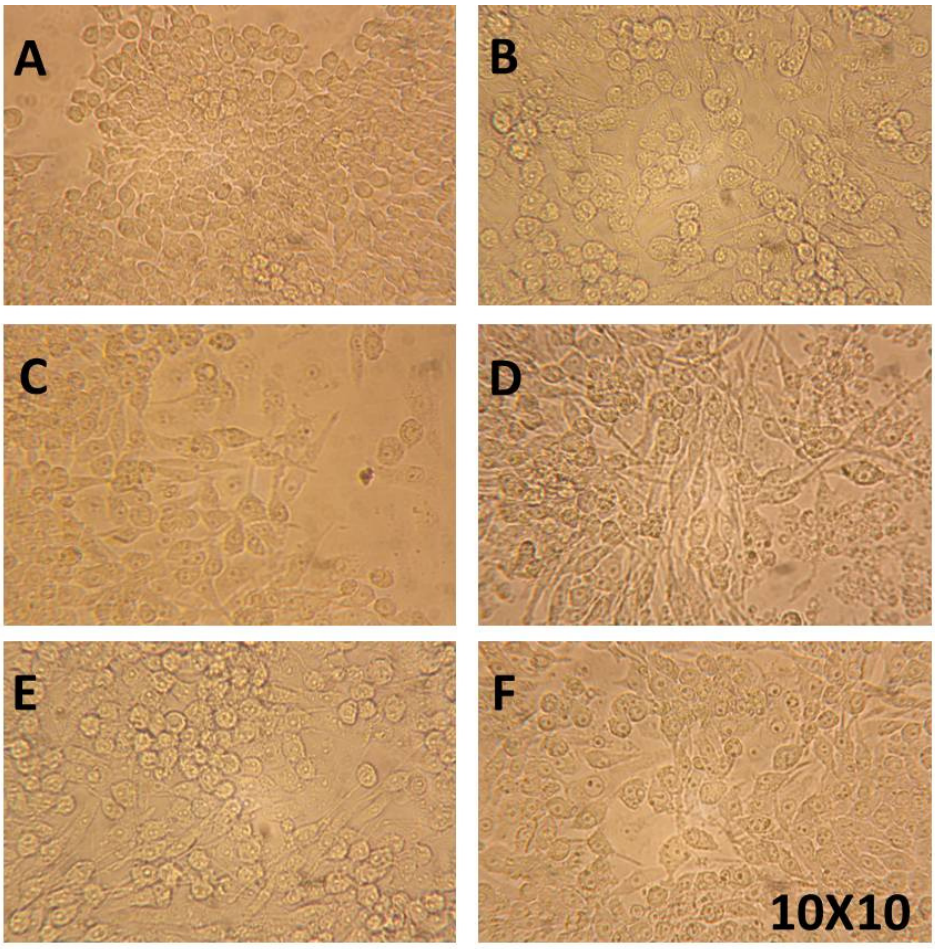

Fig 2. Cell morphology effect of TSG extracts on RAW264.7 cells. TSG extracts were incubated with medium containing LPS (0.25 μg/ml) for 20 hours. The cell images of RAW264.7 cells were observed by light microscope (Olympus). The alphabetic labels were represented as below. (A) No treatment (B) LPS treatment (C) LPS with 25 μg/ml TSG methanol extracts (D) LPS with 100 μg/ml TSG methanol extracts (E) LPS with 25 μg/ml TSG water extracts (F) LPS with 100 μg/ml TSG methanol extracts. The images were magnified to 10x10 folds.

TSG extracts suppressed the nitric oxide production induced by LPS in RAW264.7 cells

Inhibition of nitric oxide (NO) synthesis has been reported in mice fed with *Ganoderma lucidum* [13]. To examine whether TSG extract can mediate immune response, we examined the inflammatory response on NO production in LPS-treated RAW264.7 cells. We found that either TSG water or methanol extracts at high dosage such as 100 μg/ml will reduce nearly 85% of NO production in culture medium after LPS treatment (Fig. 3A and 3B). According to our estimation, the relative concentration of extracts for RAW264.7 cells treatment was relatively equal to the amount of TSG capsule administration about three capsules/ 50 kg of body weight/day. Therefore, our results *in vitro* study obtain one possible implication that even the dosage similar to the amount of one capsule/ 50 kg of body weight/day had 50% potency in the suppression of NO production (Fig. 3).

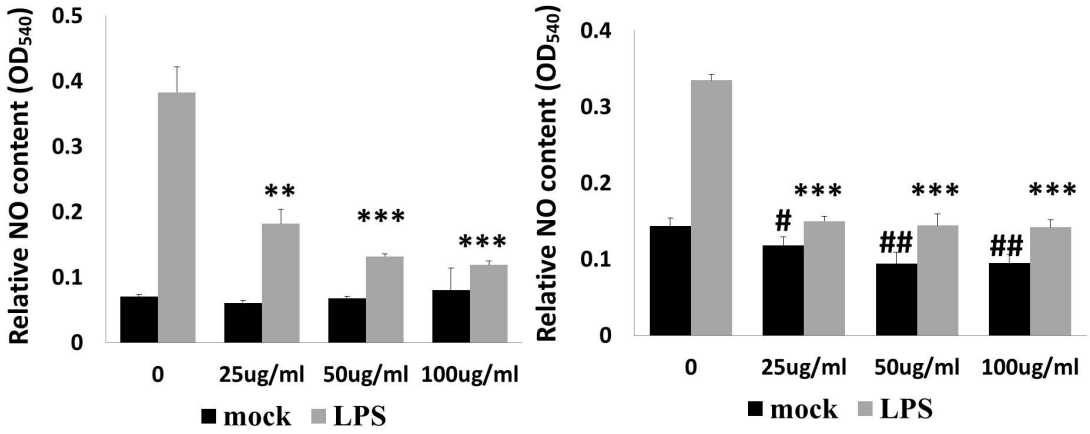

Fig 3. Effect of TSG extracts on nitric oxide production stimulated with LPS in RAW264.7 cells. Water or 80% methanol extracts of TSG were added into cell medium pretreated with LPS for 12 hours. After additional 12 hours incubation, the medium were stained with 50% Griess reagent to determine the level of nitric oxide in medium. Black bars represented as no LPS treated controls and the gray bars represented as LPS treated ones. The concentration shown in the lower panel indicated as the TSG weight to volume amount for cell treatment. The left figure was shown as the effect of TSG methanol extracts, while the right figure was shown as the effect of TSG water extracts. The experiment was performed in triplicated. # indicated as P value <0.1 (no LPS vs 25 μg/ml water extract without LPS); ## indicated as P value <0.05 (no LPS vs 50 μg/ml or 100μg/ml water extract without LPS); ** indicated as P value<0.05 (LPS vs LPS with 25 μg/ml methanol extract); *** indicated as P value<0.01 (LPS vs LPS with other concentrations of TSG extracts).

The suppression of LPS-induced NF-κB activation in THP-1 cells by TSG extracts was correlated with the reduction of NO synthesis by TSG extracts

To further characterize how TSG extract regulate nitric oxide production in RAW264.7 cells, we tried to determine whether inducible form of nitric oxide synthase (iNOS) is affected by TSG extracts. Since iNOS expression has been known to be regulated by NF-κB in LPS-stimulated RAW264.7 cells [14, 15], NF-κB reporter assay was conducted in the functional detection. As we introduced p50-based reporter into RAW264.7 cells, the transfection efficiency was too low to be detectable in the reliable level. Then we changed our experiment using human monocytic THP-1 cells easier for transient transfection. The reporter assay has found that the transfection of p50, the subunit of NF-κB, alone will stimulate the reporter activity. Addition of LPS also activates reporter activity. If cells were treated with LPS in the presence of p50, the activation will be further activated the NF-κB expression. Both types of TSG extracts (water or methanol extracts) caused the inhibitory effect on the NF-κB activation in presence of both p50 and LPS (Fig. 4). However, the reduction of reporter expression by water extracts cannot be completely suppressed the further activation. It indicated the activation pathway(s) other than NF-κB may contribute to the LPS-dependent activation of NO production. The decrease of further activation under methanol extracts treatment is more prominent than the one under the treatment of water extracts. It suggests that the reduction of NO production by TSG extracts through the inhibition of NF-κB appeared mainly from methanol extracts.

Since triterpenes of TSG should be extracted from methanol fraction, these results are consistent with recent finding about lanostane, one triterpene compound, from *Gonaderma* in the suppression of LPS-induced NO production in RAW264.7 cells [16]. Similar results were also observed in the study of ethanol extract of *Ganoderma lucidum* in the suppression of NF-κB and toll-like receptor pathways in LPS-induced BV2 microglial cells [17]. However, LZ-8 will augment NF-κB activity in human dendritic cells [18]. The fact could be explained why the inhibition of NF-κB activity under LPS and TSG water extracts less than the reduction of activity treated with LPS and TSG methanol extracts. Although the difference of suppression in NF-κB activity found in water and methanol extracts, the exact components in TSG extracting fractions remained to be determined.

Taken together, our investigation of TSG effect on inflammatory response indicated that extraction of TSG powder cannot affect the cell viability of immune cells. Only TSG methanol extracts will change cell morphology at high dosage treatment. Both extracts can effectively reduce the inflammatory response of RAW264.7 cells in NO production stimulated by LPS. The suppression might be through the NF-κB dependent activation of iNOS expression.

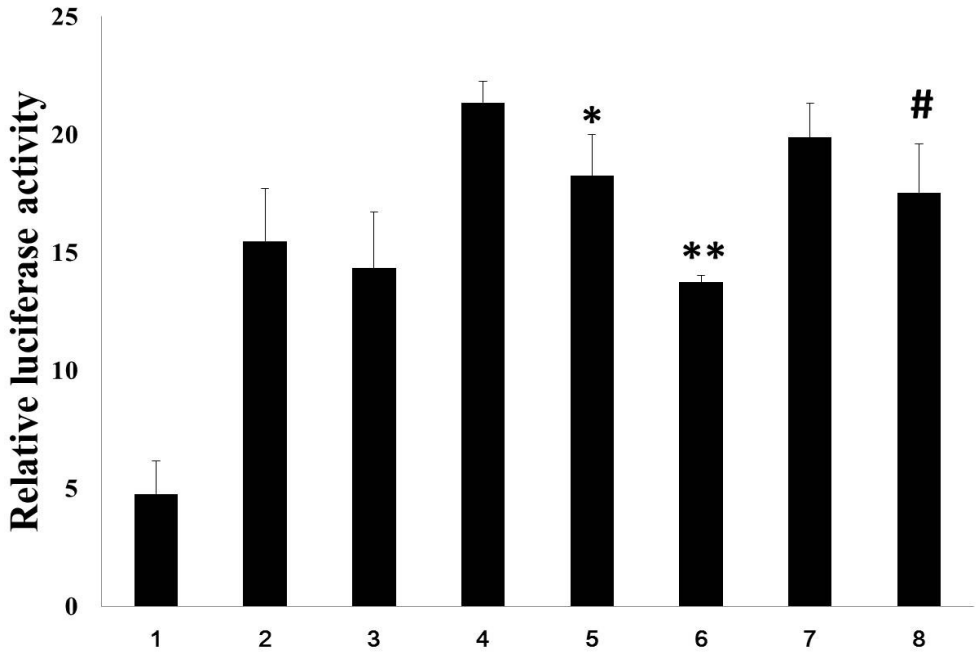

Fig 4. Effects of TSG extracts on the NF-κB activity induced by LPS or p50 NF-κB overexpression The relative luciferase activity drived by NF-κB promoter was examined in THP-1 transfected cells. CMV-p50 plasmid as well as NF-κB luciferase reporter vector was transiently transfected into cells. After one day incubation, the cells were treated with LPS as well as TSG extracts for 16-18 hours. The lysates were harvested and measured their luciferase activity normalized with CMV-drive Renilla luciferase activity in each transfection. The experiment was performed in triplicated. The number of lower panel represented as 1 (reporter without LPS), 2 (reporter and p50 expression without LPS), 3 (LPS treatment), 4 (reporter and p50 expression under LPS treatment), 5 (reporter, p50 under LPS and 400 μg/ml TSG methanol extracts), 6 (reporter, p50 under LPS and 1000 μg/ml TSG methanol extracts), 7 (reporter, p50 under LPS and 400 μg/ml TSG water extracts), and 8 (reporter, p50 under LPS and 1000 μg/ml TSG water extracts). # indicated as P value <0.1 (7 vs 8); * indicated as P value<0.1 (4 vs 5); ** indicated as P value<0.05 (4 vs 6).

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天生靈芝萃取液可透過NF-κB路徑抑制細菌脂多醣誘發小鼠RAW264.7免疫細胞株的一氧化氮生成

朱建華、葉舒平、吳慧中、邱啟銘*

銘傳大學 健康科技學院 生物科技學系 (中華民國 台灣 桃園)

中文摘要

靈芝是一種東方傳統中藥主要功效為抗癌與免疫調節作用。本篇研究針對天生靈芝產品是否具有免疫調節功效進行探討；我們採用小鼠單核球/巨噬細胞株RAW264.7 做為研究工具。研究發現天生靈芝粉末萃出液在建議用量範圍內不會影響免疫細胞存活率，但是在高濃度甲醇萃取液會造成免疫細胞型態變化。此外，靈芝水萃液與甲醇萃液均可有效抑制免疫細胞因細菌脂多醣誘發的一氧化氮釋出，這結果顯示此產品具有免疫抑制調節效果。報導基因研究發現此調控方式可能經由 NF-κB 路徑對一氧化氮生成酵素 iNOS 產生抑制作用。因此，此研究提供初步證據確定天生靈芝對免疫發炎反應具有抑制功效。

關鍵字：靈芝、免疫調節、發炎、一氧化氮
通訊作者: 邱啟銘[cmchiu@mail.mcu.edu.tw]

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