

Original Article

***Ganoderma* extract prevents albumin-induced oxidative damage and chemokines synthesis in cultured human proximal tubular epithelial cells**

Kar Neng Lai, Loretta Y. Y. Chan, Sydney C. W. Tang and Joseph C. K. Leung

Division of Nephrology, Department of Medicine, Queen Mary Hospital, The University of Hong Kong, Hong Kong

Abstract

Background. *Ganoderma lucidum* (*Ganoderma* or lingzhi) is widely used as an alternative medicine remedy to promote health and longevity. Recent studies have indicated that components extracted from *Ganoderma* have a wide range of pharmacological actions including suppressing inflammation and scavenging free radicals. We recently reported that tubular secretion of interleukin-8 (IL-8) induced by albumin is important in the pathogenesis of tubulointerstitial injury in the proteinuric state. In this study, we explored the protective effect of *Ganoderma* extract (LZ) on albumin-induced kidney epithelial injury.

Methods. Growth arrested human proximal tubular epithelial cells (PTECs) were incubated with 0.625 to 10 mg/ml human serum albumin (HSA) for up to 72 h. HSA induced DNA damage and apoptosis in PTEC in a dose- and time-dependent manner. Co-incubation of PTEC with 4–64 µg/ml LZ significantly reduced the oxidative damage and cytotoxic effect of HSA in a dose-dependent manner ($P < 0.001$). Increased release of IL-8 and soluble intercellular adhesion molecules-1 (sICAM-1) in PTEC induced by HSA was ameliorated by co-incubation with *Ganoderma* (16 µg/ml). To explore the components of LZ that exhibited most protective effect in HSA-induced PTEC damages, LZ was further separated into two sub-fractions, LZF1 (MW <30 kDa) and LZF2 (MW <3 kDa), by molecular sieving using millipore membrane. PTEC were incubated with 5 mg/ml HSA in the presence of different doses of LZF1, LZF2 or unfractionated LZ.

Results. There was no difference in the degree of protection from HSA-induced cytotoxicity or oxidative DNA damage between different fractions of LZ. However, low molecular weight LZ (<3 kDa) was most effective in reducing sICAM-1 released from

HSA-activated PTEC whereas the high molecular weight LZ (unfractionated LZ) was more effective in diminishing IL-8 production.

Conclusions. Our results suggest that *Ganoderma* significantly reduces oxidative damages and apoptosis in PTEC induced by HSA. The differential reduction of IL-8 or sICAM-1 released from HSA-activated PTEC by different components of the LZ implicates that components of *Ganoderma* with different molecular weights could play different roles and operate different mechanisms in preventing HSA-induced PTEC damage.

Keywords: chemokines; *Ganoderma*; lingzhi; proteinuria; proximal tubular epithelial cells

Introduction

Tubulointerstitial damage followed by scarring and progressive loss of renal function is the final common pathologic pathway in many forms of chronic proteinuric renal diseases. The severity of tubulointerstitial injury, which correlates with the amount of proteinuria, is a major determinant of the degree and rate of progression of renal failure. The putative role of urinary proteins, taken to reflect the degree of protein trafficking through the glomerular capillary, in inducing tubulointerstitial changes is supported by clinical observations as well as animal models of protein overload and experimental nephrotic syndrome [1]. Emerging evidence indicates that interstitial lesions induced by proteinuria may be mediated through tubular epithelial cell activation. One constant feature of proteinuric nephritis is the concomitant presence of tubulointerstitial inflammation, which is characterized by the infiltration of the interstitial space by mononuclear leucocytes, notably T-cells, monocytes and macrophages, which appear to play a key role in the subsequent evolution of tubulointerstitial inflammation

Correspondence and offprint requests to: Professor K. N. Lai, Department of Medicine, University of Hong Kong, Queen Mary Hospital, 102 Pokfulam Road, Hong Kong.
Email: knlai@hkucc.hku.hk

and fibrosis. Chemokines secreted by tubular cells almost certainly play a pivotal role in leucocyte recruitment. Monocyte chemoattractant protein-1 (MCP-1), interleukin-8 (IL-8), and RANTES are three C-C chemokines that have recently been shown to be upregulated by albumin in cultured proximal tubular cells [2–4].

Ganoderma lucidum (*Ganoderma* or lingzhi) is a natural herbal medicinal fungus that has been used in China since 100 AD. Recently, this medicinal Chinese mushroom has been demonstrated to possess tumoricidal [5] as well as immunodulatory activities [6]. *In vitro* studies show *Ganoderma* extract activates mitogen-activated protein kinase (MAPK) and inhibits lipid peroxidation and oxidative DNA damage [7,8]. Increasing evidence has been accumulated on the medicinal application of *Ganoderma* in the treatment of immunological disorders [9]. In the present study, we examine the *in vitro* effect of *Ganoderma* extract on the oxidative damage and chemokines synthesis in cultured human proximal tubular epithelial cells (PTECs) induced by albumin. The modulation of tubulo-interstitial injury in this model may have potential experimental basis to support the therapeutic potential of *Ganoderma*.

Material and methods

Preparation of water-soluble Ganoderma extract

In this study, *Ganoderma* extract (product code RLZ91; batch number 970117) was provided by Vita Green Company (Hong Kong). Briefly, to isolate water-soluble polysaccharides from *Ganoderma*, samples were mostly extracted with hot water (95–100°C). Then ethanol was added to the concentrated aqueous extract and the resulting precipitate (polysaccharide fraction) was collected by centrifugation. After cleaning, drying, ethanol extraction and vacuum concentration (60°C, 76 cmHg), a batch of crude extract of *Ganoderma* was obtained. The major components consisted of mainly polysaccharide with small amount of nucleotide, tripenoids and Ling-Zhi-8 from HPLA analysis [10,11]. A single stock of *Ganoderma* extract was prepared for all cell culture experiments. In one experiment, *Ganoderma* extract was further fractionated into three preparations (LZ, total *Ganoderma* extract; LZ F1: *Ganoderma* extract with MW <30 kDa; LZ F2 *Ganoderma* extract with MW <3 kDa) by passing through a series of millipore membranes with cut-off of different MW (Millipore, Bedford, MA, USA).

Reagents

Medium, reagents for cell culture, antibodies for cell characterization, and general chemicals were purchased from Sigma Chemicals (Poole, Paisley, UK). Human serum albumin (HSA) was obtained from CSL Laboratory (Parkville, Victoria, Australia). The endotoxin level in all albumin preparations were <10 EU/ml as determined using the QCL-1000 Limulus Amebocyte Lysate (LAL) kit (BioWhittaker Inc., Walkersville, MD, USA). Antibiotics, sera, agarose and DNA size markers were obtained from

Gibco BRL (Paisley, UK). Enzyme immunoassay kits for detection of IL-8 and soluble intracellular adhesion molecules-1 (sICAM-1) were purchased from Bender MedSystems (Vienna, Austria). Fluorescence probe for intracellular reactive oxygen species detection was from Molecular Probes Inc. (Eugene, OR, USA). All other antibodies were from Dako A/S (Glostrup, Denmark). Other chemicals were obtained from Sigma (St Louis, MO, USA).

PTEC culture

Human PTECs were isolated according to a previously described method [4]. Briefly, renal cortical tissue was obtained from kidneys removed for circumscribed tumours. Histological examination of these kidney samples revealed no renal pathology. Cortical specimens were cut into small cubes and passed through a series of mesh sieves of diminishing pore size. PTECs were collected on the 53 µm sieve, and digested with collagenase (750 U/ml) at 3°C for 15 min. Tubular cells were isolated by centrifugation and grown in a renal epithelial basal medium (REBM) consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% fetal calf serum, hydrocortisone (40 ng/ml), L-glutamine (2 mM), benzyl penicillin (100 IU/ml) and streptomycin (100 µg/ml). The cells were incubated at 37°C in 5% CO₂ and 95% air. They were characterized to be of proximal tubular origin by immunofluorescence and enzyme histochemistry: cells stained positively for cytokeratin, vimentin and alkaline phosphatase, but negatively for Tamm-Horsfall glycoprotein, factor VIII-related antigen and α-smooth muscle actin. Scanning electron microscopy demonstrated the presence of numerous apical microvilli of a rudimentary brush border with reassembly of tight junctions. Experiments were performed with cells up to the third passage, as it has been shown that there are no phenotypic changes up to this passage number [4]. In all experiments, there was a 'growth arrest' period of 48 h in serum-free medium prior to stimulation. Results were obtained from PTEC cultured from the kidney of three different donors.

Analysis of IL-8 and ICAM-1 gene expression

Gene expression of IL-8 and ICAM-1 was determined by reverse transcription and polymerase chain reaction (RT-PCR) as previously described [4]. The oligonucleotide sequences for IL-8 were: forward-ATG ACT TCC AAG CTG GCC GTG CT, reverse-TCT CAG CCC TCT TCA AAA ACT TCT; sICAM-1: forward-GAG ACC CCG TTG CCT AAA, reverse-CCG CAG GTC CAG TTC AGT; yielding amplified products of 298 bp (IL-8) and 399 bp (ICAM-1). PCR reactions were carried out in a DNA thermal cycler (MJ Research, Watertown, MA, USA), with 28–30 cycles of amplification at an annealing temperature of 55–59°C. For semi-quantification, human glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) primers were included in every reaction as an internal control. The PCR products were separated by 1.5% wt/vol agarose gels, stained with ethidium bromide (Sigma) and the gel image was captured and analysed using the Gel Doc 1000 Densitometry System and Quantity One (Bio-Rad Laboratories Ltd, Hercules, CA, USA). The product yield was expressed as a ratio to GAPDH.

Assay of IL-8 and sICAM-1 proteins in culture supernatants

PTEC were grown to confluence in six-well cell culture plates, growth-arrested and exposed to albumin (1.25–20 mg/ml) for defined time periods (6, 24 or 48 h) at 37°C in the presence or absence of *Ganoderma* extract. The concentration of albumin and the duration of experiments were selected based on our previous data [4]. At the end of the experiment, cell number was counted and supernatants were collected and stored at –70°C until further use. Detection of IL-8 and sICAM-1 levels in culture supernatants was carried out on commercially available assay kits. The detection sensitivity and intra-assay CV is 11 pg/ml ± 3.8% for IL-8 and 0.63 ng/ml ± 6.8% for sICAM-1.

Quantification of proliferation and viability of PTEC cultured with Ganoderma extract

The optimal or sub-toxic dose of *Ganoderma* extract was determined by cell viability measured by a commercial colorimetric kit (Chemicon International, Temecula, CA, USA) based on cleavage of the tetrazolium salt WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt]. Briefly, growth arrested PTEC were seeded into 96-well plates (0.25×10^5 cells per well) before being exposed to *Ganoderma* extract for 48 h. WST-1 reagent was then added and incubated at 37°C for a further 2 h. The absorbance was measured using 450 nm as the primary wavelength and 600 nm as the reference wavelength. Results were expressed as percentage changes in absorbance compared with that of the medium control.

Determination of apoptosis and oxidative damage of PTEC

The level of apoptosis was determined by a TUNEL assay carried out with the ApopTaq Fluorescein *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA) following the manufacturer's protocol. Briefly, cells were grown on Lab-Tak chamber slides (Nalgen Nunc International, Naperville, IL, USA) and fixed with 1% paraformaldehyde. The fixed cells were subsequently incubated in TdT equilibration buffer followed by TdT reaction buffer. After washing, the cells were incubated with anti-digoxigenin-fluorescein. Slides were mounted and the apoptotic cells with green fluorescence were visualized under a fluorescence microscope with a FITC filter (excitation 490 nm, emission 520 nm). For a positive control, camptothecin was added to the media (1:1000) for 24 h before analysis. In addition, activation of caspase 3 was determined using the caspase 3 activity fluorometric immunosorbent enzyme assay kit (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's protocol.

The oxidative damage of DNA in PTEC following exposure to *Ganoderma* extract was determined by a fluorogenic OxyDNA Assay kit (Calbiochem–Novabiochem Co., La Jolla, CA, USA). The OxyDNA Assay is an *in vitro* fluorescent protein binding method used for the detection of oxidative DNA in fixed and permeabilized cells. It uses 8-oxoguanine (as part of the oxidized nucleotide 8-oxoguanosine), which is a sensitive marker formed during free radical damage to DNA and is a sensitive and specific

indicator of oxidative DNA damage. The assay is based on the binding of a FITC-labelled probe to the 8-oxoguanine moieties present in the DNA of the fixed and permeabilized cells. Fluorescence was visualized by fluorescence microscopy according to the protocol recommended by the manufacturer.

Measurement of intracellular reactive oxygen species (ROS)

The intracellular formation of ROS was detected by the fluorescence probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) (Molecular Probes Inc., Eugene, OR, USA). PTECs (1×10^6 /ml) were cultured in the presence of different doses of HSA (4–16 mg/ml) or 16 mg/ml HSA with or without the addition of *Ganoderma* extract. The cells were incubated for 30 min and then loaded with 0.1 µg/ml CM-H₂DCFDA. The cells were placed on ice. ROS production, expressed as mean fluorescence intensity (MFI), was immediately measured by flow cytometry using a Coulter EPICS XL analyzer (Coulter Electronic, Miami, FL, USA). Results were expressed as MFI% of control cells incubated with culture medium alone.

Statistical analysis

All data (from five experiments) were expressed as means ± SD. Inter-group differences for continuous variables were assessed by the unpaired *t*-test. The cell viability of PTEC following exposure to different concentrations of *Ganoderma* extract was analyzed with multivariate ANOVA for repeated measures. Statistical analysis was performed using statistical software (Statview, SAS Intelligence, Cary, NC, USA). All *P*-values quoted are two-tailed and the significance is defined as *P* < 0.05.

Results

Viability of PTEC cultured with Ganoderma

Figure 1A showed the cell viability measured by cell proliferation in PTEC following incubation of two concentrations of HSA. Incubation of PTEC with HSA at 2.5 mg/ml or 5 mg/ml reduced the cell proliferation by 68 and 73%, respectively. *Ganoderma* extract restored the cell viability in a dose-dependent manner (*P* < 0.001). The cell viability improved to 85–95% of control values at a *Ganoderma* concentration of 16 µg/ml. It is noteworthy that cell viability fell by 11% in PTEC incubated with *Ganoderma* extract alone at a concentration of 64 µg/ml (without HSA). Cell viability was reduced by 18, 24, 50 and 17% when PTEC were incubated with *Ganoderma* extract alone at a concentration of 128, 256, 512 and 1026 µg/ml, respectively (not shown in figure). Figure 1B shows the micrographs of trypan blue stained PTEC cultured with different concentrations of *Ganoderma* extract upon prior incubation of 0, 2.5 or 5 mg/ml of HSA. The findings paralleled those of WST assay showing less than 20% of the cultured PTEC demonstrating uptake of the dye when incubated with HSA at 2.5 or 5 mg/ml.

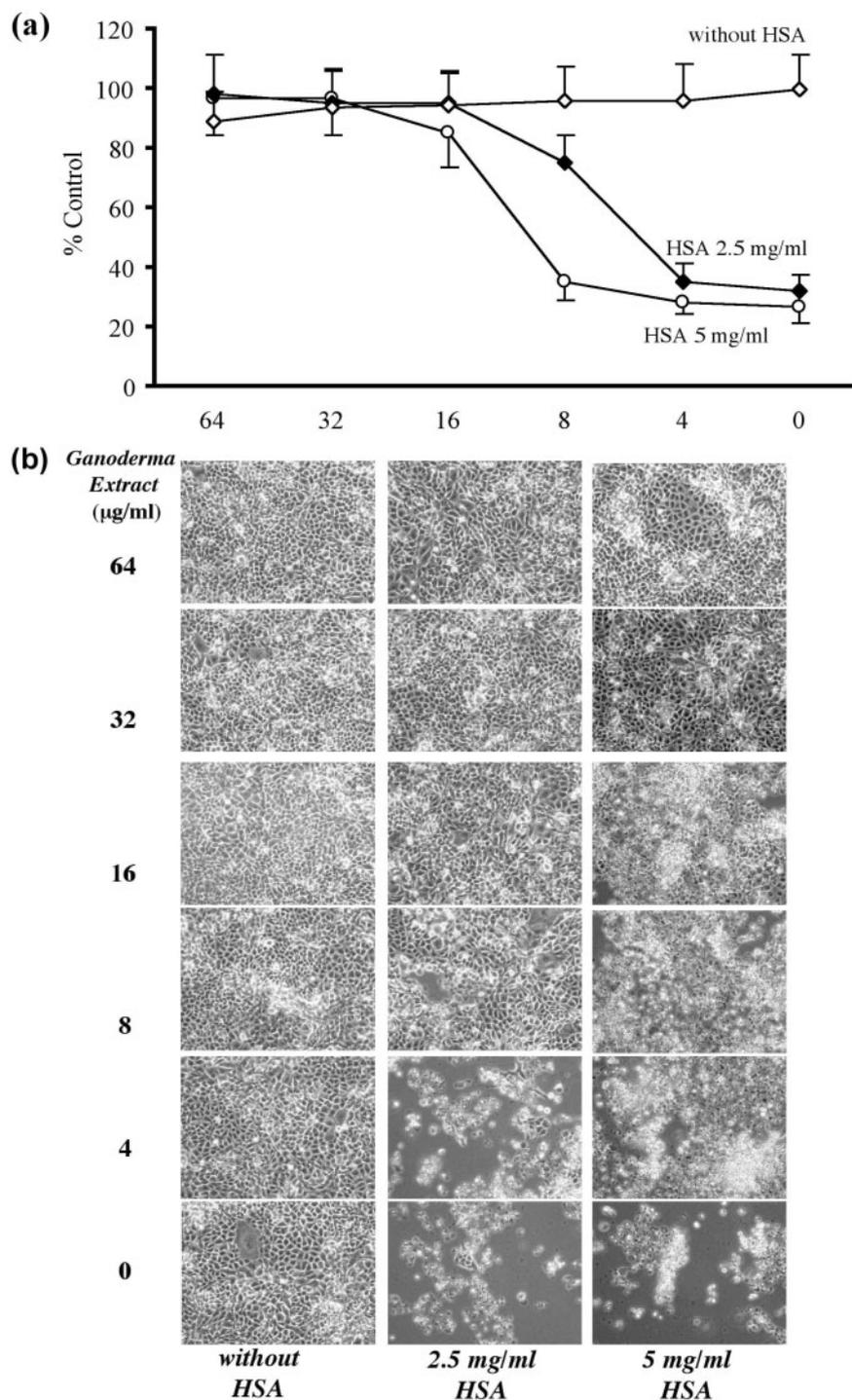


Fig. 1. (A) Cell proliferation assay in PTEC incubated with HSA (for 48h) determined by WST kit. *Ganoderma* extract increased the cell viability. (B) Micrographs showing reduced cell viability of PTEC following acute exposure to HSA for 48h.

The dye uptake and cell morphology improved with addition of *Ganoderma* extract.

Effect of Ganoderma extract on apoptosis and oxidative damage of PTEC induced by albumin

Following exposure to HSA at either 2.5 or 5 mg/ml, PTEC exhibited significant apoptotic changes

demonstrated by TUNEL (Figure 2A). The apoptotic changes were abolished with addition of *Ganoderma* extract at a concentration of 16 µg/ml. Activation of caspase 3 was observed in PTEC incubated with HSA for 24h. The caspase 3 activation was significantly reduced in the presence of *Ganoderma* extract at concentrations of 8 and 16 µg/ml ($P < 0.001$ vs PTEC incubated without *Ganoderma*) (Figure 2B).

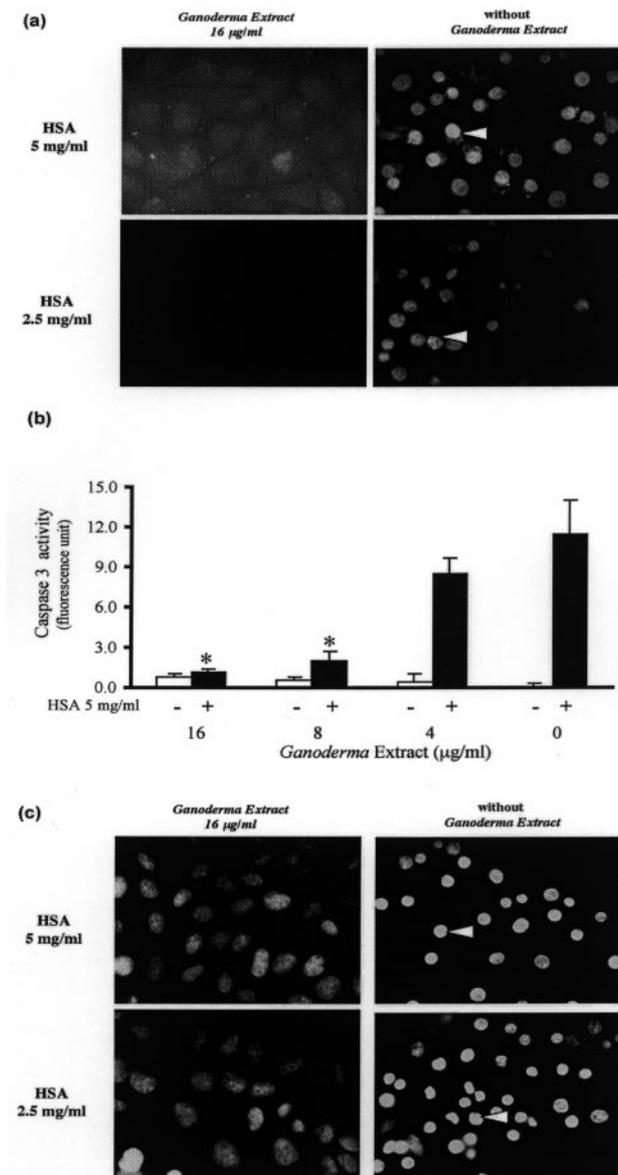


Fig. 2. (A) Apoptosis assay by TUNEL showing increased apoptotic changes in PTEC incubated with HSA (for 24 h). The apoptotic changes were minimized with *Ganoderma* extract (LZ). (B) Activation of caspase 3 was observed in PTEC incubated with HSA (for 24 h) and this caspase 3 activation was significantly reduced (* $P < 0.001$ vs PTEC incubated without *Ganoderma*) in the presence of 8 and 16 µg/ml of *Ganoderma* extract. (C) Oxidative damage of DNA in PTEC incubated with HSA (for 24 h). The oxidative DNA damage was minimized with *Ganoderma* extract.

Similarly, *Ganoderma* extract at a concentration of 16 µg/ml reduced the oxidative damage of DNA in PTEC incubated with two cytotoxic concentrations of HSA (Figure 2C). The cytoprotective effect of *Ganoderma* extract on the oxidative damage of DNA in PTEC induced by HSA was dose-dependent (Figure 3A) while incubation of PTEC with

Ganoderma extract alone (at a concentration between 4–16 µg/ml) showed no oxidative damage of DNA (Figure 3B) or induction of intracellular ROS generation in PTEC (Figure 4).

Effect of Ganoderma extract on IL-8 and sICAM-1 expression from PTEC induced by albumin

There was a significant increase of ICAM-1 expression (Figure 5A) with a 7-fold increase in sICAM-1 release from PTEC following exposure to HSA at 5 mg/ml (Figure 5B). *Ganoderma* extract reduced the ICAM-1 mRNA expression and sICAM-1 release from PTEC induced by HSA in a dose-dependent manner up to a concentration of 16 µg/ml, above which no further reduction was observed. Incubation of PTEC with *Ganoderma* extract alone (at a concentration between 2–16 µg/ml) showed no significant induction of ICAM-1 mRNA expression or sICAM-1 release from PTEC. Two-fold and 3.5-fold increases in sICAM-1 release, but not ICAM-1 mRNA expression, were detected with *Ganoderma* extract at a concentration of 32 or 64 µg/ml, respectively. Upon exposure to HSA at 5 mg/ml, there was a significant increase in IL-8 mRNA expression and an 18-fold increase in IL-8 release from PTEC (Figure 6B). Again, *Ganoderma* extract reduced the IL-8 mRNA expression and IL-8 release from PTEC induced by HSA in a dose-dependent manner up to a concentration of 32 µg/ml, above which no further reduction was observed. Incubation of PTEC with *Ganoderma* extract alone (at a concentration between 2–16 µg/ml) showed no induction of IL-8 mRNA expression or IL-8 release from PTEC although a 2-fold increase was detected with *Ganoderma* extract at a concentration of 32 µg/ml.

Pathophysiological actions of different fractions of Ganoderma separated by molecular weight

We then fractionated the *Ganoderma* extract into three preparations (LZ, total *Ganoderma* extract; LZ F1, *Ganoderma* extract with MW <30 kDa; LZ F2, *Ganoderma* extract with MW <3 kDa) (Figure 7A). All three fractions of *Ganoderma* extract restored the cell viability in a dose-dependent manner with cell viability improved to 77–88% of control values at a concentration of 16 µg/ml (Figure 7B). Similarly, all three fractions at identical concentration exhibited comparable reduction of ROS generation in PTEC induced by preincubation with HSA (Figure 8). Incubation with all three fractions of *Ganoderma* extract at either 16 or 32 µg/ml significantly reduced the sICAM-1 release from PTEC induced by HSA (Figure 9A). The reduction with either LZ F1 or LZ F2 fractions was lower than that of unfractionated LZ. In contrast, reduction of IL-8 release was only observed in PTEC that were incubated with unfractionated *Ganoderma* extract at a dose-dependent manner (Figure 9B). However, similar reduction of IL-8 release from PTEC was not observed with either LZ F1 or LZ F2 fractions.

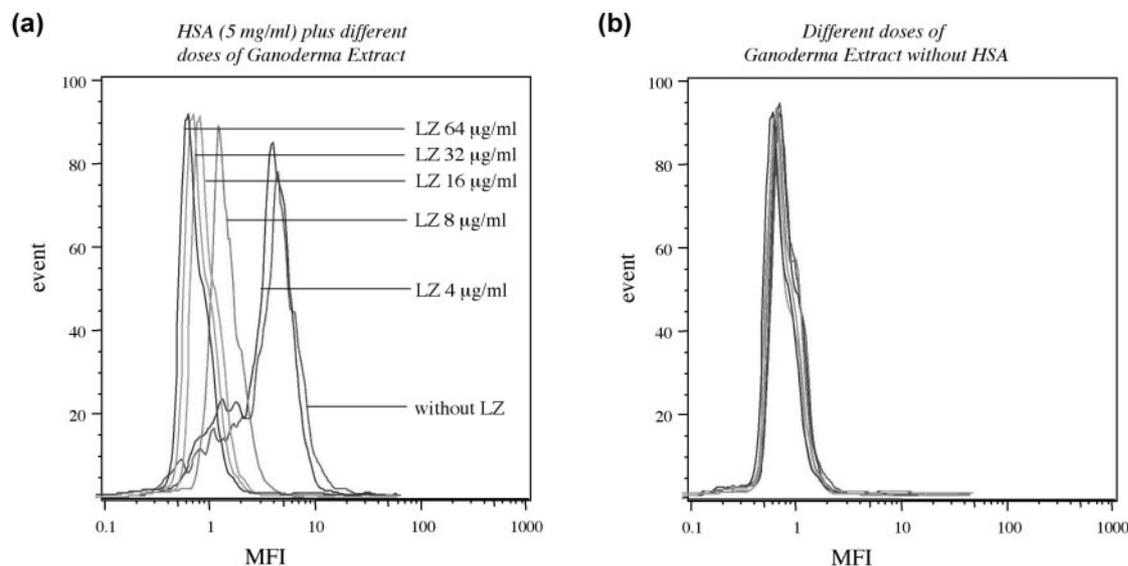


Fig. 3. (A) Representative cytometric results of ROS generation as determined by flow cytometry. The cytoprotective effect of *Ganoderma* extract (LZ) on the ROS released in PTEC induced by HSA (24h incubation) was dose-dependent. (B) Incubation of PTEC with *Ganoderma* extract alone (at a concentration between 4–16 µg/ml) showed no induction of ROS generation.

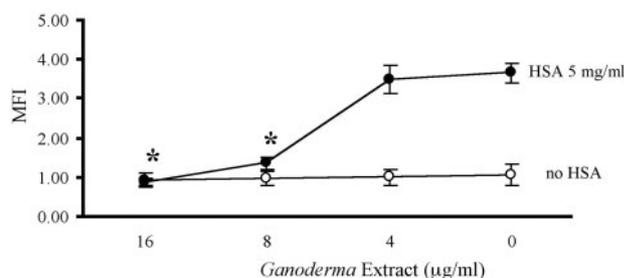


Fig. 4. ROS generation as determined by flow cytometry. The results represent mean \pm SD of five separate experiments. * $P < 0.001$ when compared with PTEC incubated with HSA (for 24h) in the absence of *Ganoderma*.

Discussion

The fungus *Ganoderma lucidum*—also known as ‘Lingzhi’ in Chinese, ‘Reishi’ in Japanese, and ‘Youngzhi’ in Korean—is a member of the genus *Ganoderma* and has been traditionally used as a popular herbal medicine for the promotion of health in the Orient. The genus *Ganoderma*, however, was established in the west by a Finnish botanist, Karsten, in 1881 [5], and more than 120 species have been reported in the world since then. In addition to *G. lucidum* (red), other members of the genus *Ganoderma* that are commonly known to possess medicinal/nutritional values include *G. applanatum* (brown), *G. tsugae* (red-brown), *G. sinense* (dark purple to black) and *G. capense* (dark red). In the present study, we utilize a primary PTEC culture to demonstrate the presence of reno-protective compounds in the Chinese medicinal mushroom, *G. lucidum*. Incubation with *Ganoderma* extract results in improved cell viability and reverses the cytotoxicity

induced by HSA. More importantly, the *Ganoderma* extract also protects the PTEC from apoptosis and DNA damage induced by exposure to HSA. Furthermore, the ability of *Ganoderma* extract to reduce the release of sICAM-1 and IL-8 from PTEC upon exposure to HSA suggests that *Ganoderma* extract comprises a rich source of immunomodulatory compounds that possess anti-inflammatory actions.

Many biological effects of *Ganoderma* have recently been reported in scientific literature. Many of these suggest that *Ganoderma* has both immunoreactive and immunomodulatory effects. It exhibits anti-allergic properties to inhibit histamine release from mast cells [12]. *Ganoderma* also reduces production of IgG antibodies and suppresses IL-2 and ICAM-1 release from T cells [13,14]. On the other hand, *Ganoderma* enhances splenic natural killer cell activity and serum interferon production in mice [15], and induces neuronal differentiation of PC12 cells through activation of MAP kinases [7]. The bidirectional effect of *Ganoderma* in autoimmunity is also supported by our findings. While *Ganoderma* suppresses the inflammatory and apoptotic activities in PTEC induced by HSA within a dose range, incubation with *Ganoderma* alone at a higher concentration induces cytotoxicity and release of inflammatory mediators. These findings strongly indicate the necessity to determine a therapeutic dose for specific anti-inflammatory action.

During the past two decades, modern research has revealed that *Ganoderma* contains a variety of chemical ingredients, including triterpenes, polysaccharides, nucleosides, steroids, fatty acids, alkaloids, proteins, peptides, amino acids, and inorganic elements [11]. Among these ingredients, triterpenes and polysaccharides have attracted considerable attention as they have been shown to possess diverse and potentially significant pharmacological activities.

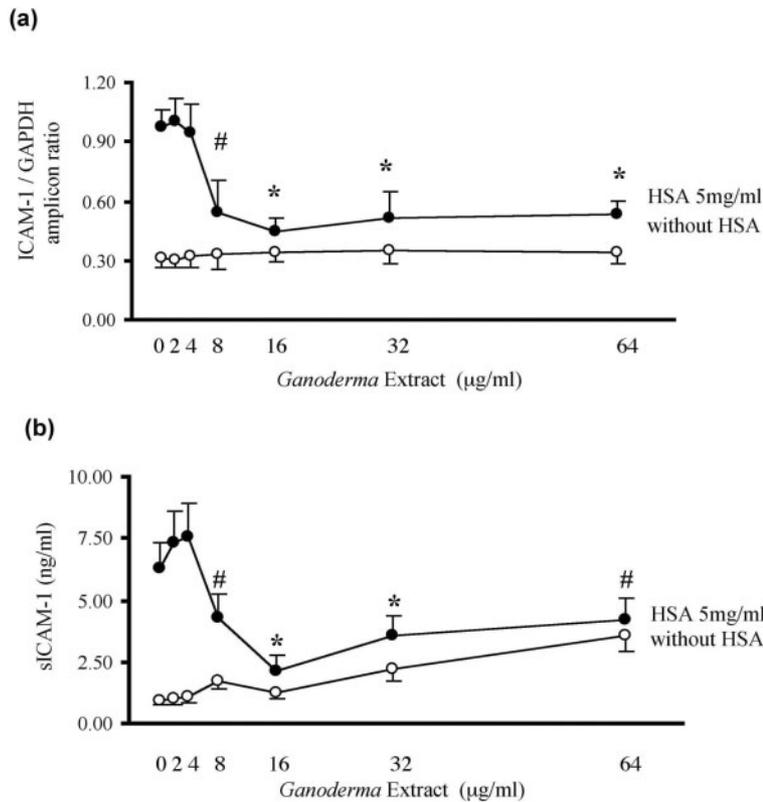


Fig. 5. ICAM-1 mRNA expression at 6h (A) and sICAM-1 protein synthesis (B) at 24h in supernatant from PTEC cultured with HSA in the absence or presence of different concentrations of *Ganoderma*. The results represent mean \pm SD of five separate experiments. * $P < 0.001$ when compared with PTEC incubated with HSA in the absence of *Ganoderma*. # $P < 0.05$ when compared with PTEC incubated with HSA in the absence of *Ganoderma*.

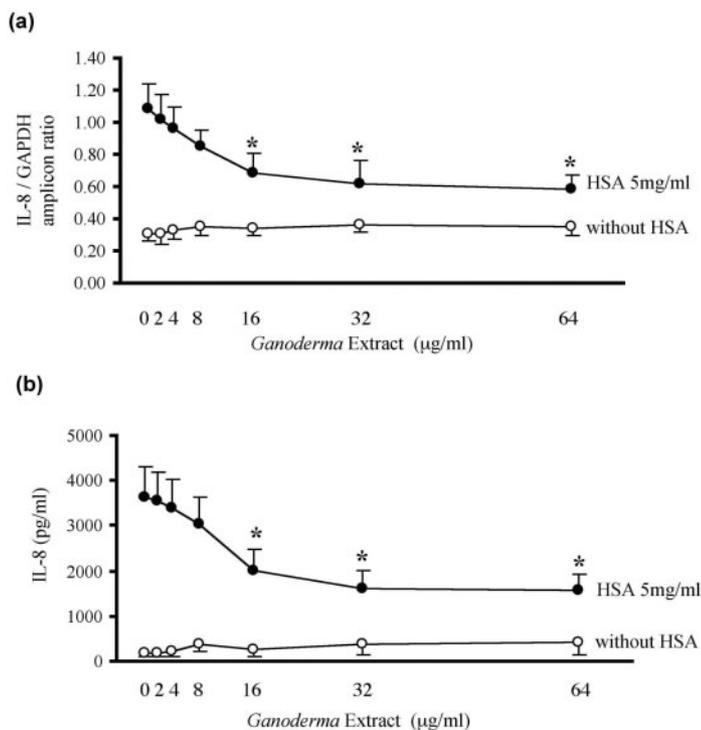


Fig. 6. IL-8 mRNA expression at 6h (A) and IL-8 protein synthesis (B) at 24h in supernatant from PTEC cultured with HSA in the absence or presence of different concentrations of *Ganoderma*. The results represent mean \pm SD of five separate experiments. * $P < 0.001$ when compared with PTEC incubated with HSA in the absence of *Ganoderma*.

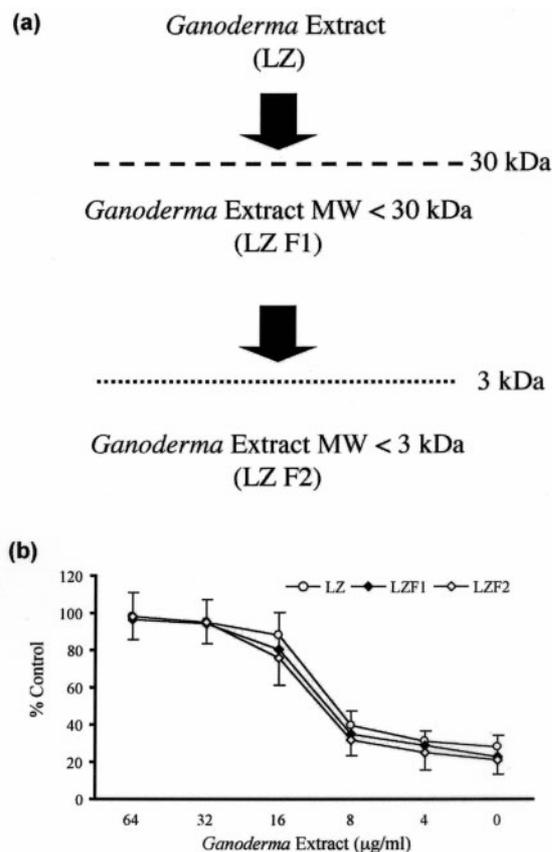


Fig. 7. (A) Fractions of *Ganoderma* extract separated by molecular weight. (B) The three fractions expressed similar protective effect on albumin-induced cytotoxicity on PTEC.

Our water-soluble preparation of *Ganoderma* extract contains many bioactive ingredients (mainly polysaccharides and some triterpenes). Three fractions of *Ganoderma* extract were prepared according to the molecular weight of the ingredients. All three fractions restored the cell viability in a dose-dependent manner and exhibited comparable reduction of the oxidative damage of DNA in PTEC induced by preincubation with HSA at identical concentrations of *Ganoderma* extract. Incubation with all three fractions of *Ganoderma* extract at either 16 or 32 µg/ml significantly reduced the sICAM-1 release from PTEC induced by HSA. However, the reduction with either LZ F1 or LZ F2 fractions was significantly lower than that of unfractionated LZ. These findings suggest the bioactivities against cytotoxicity or oxidative damage of DNA and the anti-inflammatory effect on sICAM-1 release reside in ingredients with low molecular weight <3 kDa. *Ganoderma* extract contains different low molecular weight components including monosaccharides, oligosaccharide, triterpenes, vitamins and selenium. Of particular interest, the triterpenes have been reported to possess significant anti-oxidative activity [16]. In contrast, the bioactivity against the neutrophil chemoattractant effect of IL-8 lies in ingredients with molecular weight higher than 30 kDa. Our findings in PTEC illustrate the diverse and

potentially significant pharmacological activities of the extensive array of chemical ingredients in *Ganoderma*. Peak profiling (fingerprint) of different saccharides using the high-performance thin-layer chromatography and/or automated multiple development are likely the next step forward for screening the biological activities of different ingredients in *Ganoderma* [17].

Our present data show that *Ganoderma* extract at concentrations of 32 and 64 µg/ml slightly upregulate the sICAM-1 release by PTEC. The increased release of sICAM-1 was not due to the upregulation of ICAM-1 mRNA expression. This increased release of sICAM-1 could be due to the increased cleavage of cell surface ICAM-1. However, the precise mechanism of this increased cleavage remains unknown. It has been reported that *Ganoderma* extract activates the MAPK in pheochromocytoma [7]. The extracellular signal-regulated kinase (ERK1/2) has been implicated in the cleavage of groups of transmembrane proteins [18]. It could be possible that high concentrations of *Ganoderma* extract increase the surface ICAM-1 cleavage through activation of the MAPK.

Ganoderma extract at concentration of ≥8 µg/ml significantly reduces ROS generation by PTEC exposed to HSA (5 mg/ml). However, higher concentration (≥16 µg/ml) is needed to reduce the sICAM-1 or IL-8 release as well as for maintaining the viability of PTEC cultured with HSA. Our data suggest that the protection of *Ganoderma* extract in PTEC against albumin-induced injury is not totally dependent on suppression of ROS generation. We and others have previously demonstrated that activation of NF-κB in PTEC is an important downstream event mediated by albumin [3,4]. We speculate that other ROS-independent signalling mediators could also participate in albumin-induced NF-κB activation. Higher concentration of *Ganoderma* extract is needed to suppress this ROS-independent NF-κB activation. Further experiments are needed to confirm this hypothesis.

There has been increasing evidence linking excessive protein trafficking through the glomerulus and progressive renal tubulointerstitial inflammation leading to chronic renal failure. A hallmark of inflammation is the invasion into injured tissue by activated leucocytes. This step is critically dependent on the rapid expression of chemokines. Data are already available to show that overexposure to filtered proteins upregulates tubular expression of pro-inflammatory mediators such as IL-8 [4] and macrophage migration inhibitory factor [19]. Apparently, the involvement of infiltrating inflammatory cells in the glomeruli and renal interstitium is important in mediating tubular injury and renal fibrosis. As a consequence, resident renal cells can become activated. In recent years, much attention has focused on the role of PTEC in the orchestration of inflammatory cell infiltration and interstitial fibrosis. Mediators released by infiltrating cells are directly responsible for the activation of proximal tubular cells, which in turn may intensify the inflammatory process via local production of various mediators which attract

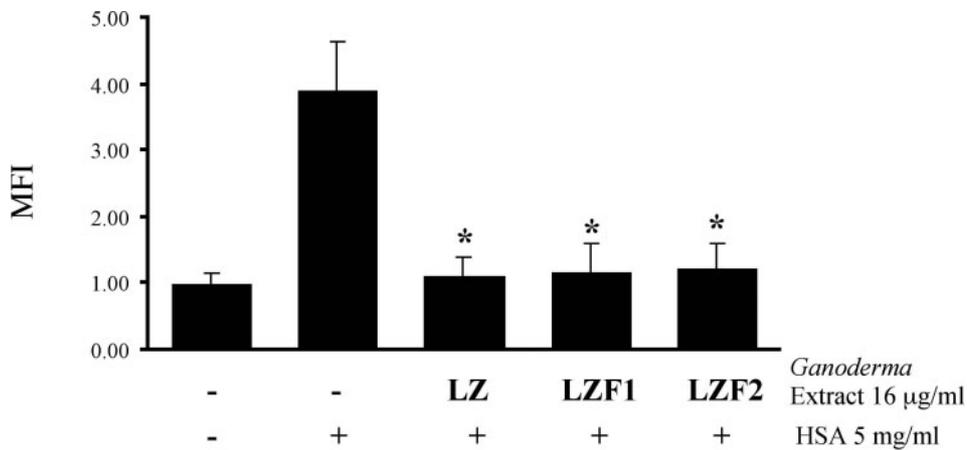


Fig. 8. Fractions of *Ganoderma* extract separated by molecular weight expressed similar protective effect on albumin-induced ROS generation (48 h incubation) in PTEC. The results represent mean \pm SD of five separate experiments. * $P < 0.001$ when compared with PTEC incubated with HSA in the absence of *Ganoderma*.

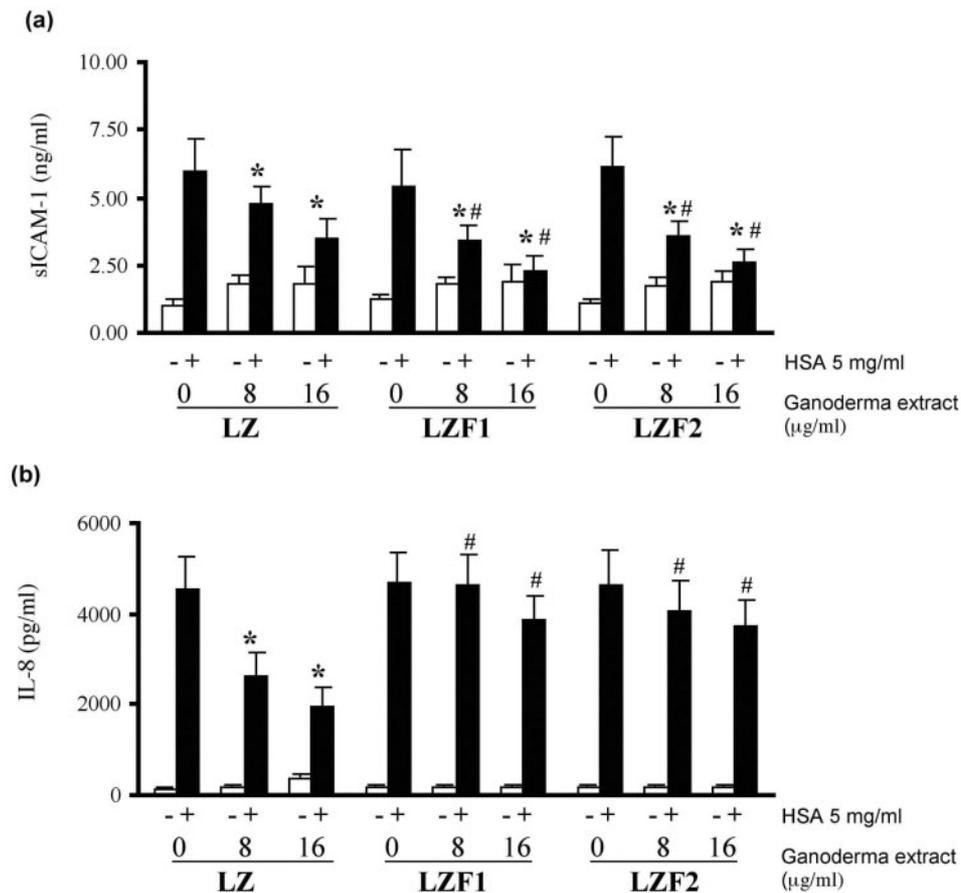


Fig. 9. (A) sICAM-1 protein synthesis at 24 h in supernatant from PTEC cultured with HSA in the absence or presence of different fractions of *Ganoderma*. The results represent mean \pm SD of five separate experiments. * $P < 0.001$ when compared with PTEC incubated with HSA in the absence of *Ganoderma* extract. # $P < 0.05$ when compared with PTEC incubated with HSA in the presence of the same concentration of unfractionated *Ganoderma* extract (LZ). (B) IL-8 protein synthesis at 24 h in supernatant from PTEC cultured with HSA in the absence or presence of different fractions of *Ganoderma*. The results represent mean \pm SD of five separate experiments. * $P < 0.001$ when compared with PTEC incubated with HSA in the absence of *Ganoderma* extract. # $P < 0.05$ when compared with PTEC incubated with HSA in the presence of the same concentration of unfractionated *Ganoderma* extract (LZ).

more inflammatory cells. The outcome of this chain reaction is the generation of a positive feedback loop of activation, leading to the overproduction of extracellular matrix components resulting in fibrosis and

ultimately loss of tissue integrity and renal failure. Treatment options for tubulointerstitial injury related to proteinuric state remain limited other than blockade of the renin-angiotensin system aiming to reduce

intra-renal pressure and proteinuria. As most available treatment is only targeted at preventing the development of renal failure rather than towards the primary etiology, long-term therapy is frequently required. Treatment with few side-effects is preferable. Ingredients purified from medicinal herbs are distinctly attractive with the notion that side-effects are fewer with natural compounds. Prevention of chronic renal failure had previously been reported with compounds purified from medicinal herbs [20]. Our present *in-vitro* study provides data suggesting the therapeutic potential of *Ganoderma* to ameliorate the tubulointerstitial injury related to proteinuric state.

In summary, this study is the first to show that *Ganoderma* extract can reduce the cytotoxicity, apoptosis, DNA damage and release of chemokines in PTEC stimulated by human albumin in an experimental setting mimicking proteinuric state. The renoprotective effect appears to be dose-related and a higher dose may induce pro-inflammatory effect. Currently, therapeutic interventions are primarily aimed at reducing glomerular protein ultrafiltration and controlling systemic hypertension. It is conceivable that future therapeutic strategies may be targeted at the pathobiologic processes that lie further downstream, namely the activation of tubular epithelial cells and their interaction with infiltrating leucocytes. Understanding the therapeutic potential of *Ganoderma* extract and its bioactivities is likely to yield novel insight into the development of new anti-inflammatory agents.

Acknowledgements. The study was partly supported by the Research Grant Committee (Hong Kong SAR) [HKU7424/03M]. J.C.K. Leung is supported by the L&T Charitable Fund and INDOCAFE.

Previously presented in abstract form at the American Society of Nephrology Annual Scientific Meeting, St Louis, MO, 29 October–21 November 2004.

Conflict of interest statement. None declared.

References

1. Eddy AA. Interstitial nephritis induced by protein-overload proteinuria. *Am J Pathol* 1989; 135: 719–733
2. Wang Y, Chen J, Chen L, Tay YC, Rangan GK, Harris DC. Induction of monocyte chemoattractant protein-1 in proximal tubule cells by urinary protein. *J Am Soc Nephrol* 1997; 8: 1537–1545
3. Zoja V, Donadelli R, Colleoni S *et al.* Protein overload stimulates RANTES production by proximal tubular cells depending on NF- κ B activation. *Kidney Int* 1998; 53: 1608–1615
4. Tang S, Leung JCK, Abe K *et al.* Albumin stimulates interleukin-8 expression in proximal tubular epithelial

- cells: an in vitro and in vivo study. *J Clin Invest* 2003; 111: 515–527
5. Wang G, Zhang J, Mizuno T *et al.* Antitumor active polysaccharides from the Chinese mushroom *Songshan lingzhi*, the fruiting body of *Ganoderma tsugae*. *Biosc Biotec Biochem* 1993; 57: 894–900
6. Kim RS, Kim HW, Kim BK. Suppressive effects of *Ganoderma lucidum* on proliferation of peripheral blood mononuclear cells. *Mol Cells* 1997; 7: 52–57
7. Cheung WM, Hui WS, Chu PW, Chiu SW, Ip NY. *Ganoderma* extract activates MAP kinases and induces the neuronal differentiation of rat pheochromocytoma PC12 cells. *FEBS Lett* 2000; 486: 291–296
8. Lee JM, Kwon H, Jeong H *et al.* Inhibition of lipid peroxidation and oxidative DNA damage by *Ganoderma lucidum*. *Phytotherapy Res* 2001; 15: 245–249
9. Lai NS, Lin RH, Lai RS, Kun UC, Leu SC. Prevention of autoantibody formation and prolonged survival in New Zealand Black/New Zealand White F₁ mice with an ancient Chinese herb, *Ganoderma tsugae*. *Lupus* 2000; 10: 461–465
10. Kino K, Yamashita A, Yamaoka K *et al.* Isolation and characterization of a new immunomodulatory protein, Ling Zhi-8, from *Ganoderma lucidum*. *J Biol Chem* 1989; 264: 472–478
11. Huie CW, Di X. Chromatographic and electrophoretic methods for Lingzhi pharmacologically active components. *J Chromatogr B* 2004; 812: 241–257
12. Tasaka K, Mio M, Izushi K, Akagi M, Makino T. Anti-allergic constituents in the culture medium of *Ganoderma lucidum*. The inhibitory effect of cyclooctasulfur on histamine release. *Agents Actions* 1988; 23: 157–160
13. Lei LS, Lin ZB. Effects of *Ganoderma* polysaccharides on T cell subpopulations and production of interleukin 2 in mixed lymphocyte response. *Yao Hsueh Hsueh Pao* 1992; 27: 331–335
14. Haak Frensch M, Kino K, Sone T, Jardieu P. Ling Zhi-8: a novel T cell mitogen induces cytokine production and upregulation of ICAM-1 expression. *Cell Immunol* 1993; 150: 101–113
15. Won SJ, Lin MT, Wu WL. *Ganoderma tsugae* mycelium enhances splenic natural killer cell activity and serum interferon production in mice. *Jpn J Pharmacol* 1992; 59: 171–176
16. Lin ZB. Focus on anti-oxidative and free radical scavenging activity of *Ganoderma lucidum*. *J Appl Pharmacol* 2004; 12: 133–137
17. Di X, Chan KK, Leung HW, Huie CW. Fingerprint profiling of acid hydrolyzates of polysaccharides extracted from the fruiting bodies and spores of Lingzhi by high-performance thin-layer chromatography. *J Chromatogr A* 2003; 1018: 85–95
18. Umata T, Hirata M, Takahashi T *et al.* A dual signaling cascade that regulates the ectodomain shedding of heparin-binding epidermal growth factor-like growth factor. *J Biol Chem* 2001; 276: 30475–30482
19. Tang S, Leung JCK, Tsang AW, Lan HY, Chan TM, Lai KN. Transferrin up-regulates chemokine synthesis by human proximal tubular epithelial cells: Implication on mechanism of tubuloglomerular communication in glomerulopathic proteinuria. *Kidney Int* 2002; 61: 1655–1665
20. Zhang JH, Li LS, Zhang M. Clinical effects of rheum and captopril on preventing progression of chronic renal failure. *Chin Med J* 1990; 103: 788–793

Received for publication: 14.9.05

Accepted in revised form: 29.12.05