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Protective effect of *Phellinus linteus* polysaccharide extracts against thioacetamide-induced liver fibrosis in rats: a proteomics analysis

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Abstract

Background: The hepatoprotective potential of *Phellinus linteus* polysaccharide (PLP) extracts has been described. However, the molecular mechanism of PLP for the inhibition of liver fibrosis is unclear. This study aims to investigate the molecular protein signatures involved in the hepatoprotective mechanisms of PLP *via* a proteomics approach using a thioacetamide (TAA)-induced liver fibrosis rat model.

Methods: Male Sprague–Dawley rats were divided into three groups of six as follows: Normal group; TAA group, in which rats received TAA only; and PLP group, in which rats received PLP and TAA. Liver fibrosis was induced in the rats by repeated intraperitoneal injections of TAA at a dose of 200 mg/kg body weight twice a week for 4 weeks. PLP was given orally at a dose of 50 mg/kg body weight twice a day from the beginning of the TAA treatment until the end of the experiment. The development of liver cirrhosis was verified by histological examination. Liver proteomes were established by two-dimensional gel electrophoresis. Proteins with significantly altered expression levels were identified by matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry and the differentially expressed proteins were validated by immunohistochemical staining and reverse transcription polymerase chain reaction.

Results: Histological staining showed a remarkable reduction in liver fibrosis in the rats with PLP treatment. A total of 13 differentially expressed proteins including actin, tubulin alpha-1C chain, preprohaptoglobin, hemopexin, galectin-5, glutathione S-transferase alpha-4 (GSTA4), branched chain keto acid dehydrogenase hterotetrameric E1 subunit alpha (BCKDHA), glutathione S-transferase mu (GSTmu); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); thiosulfate sulfurtransferase (TFT); betaine-homocysteine S-methyltransferase 1 (BHMT1); quinoid dihydropteridine reductase (QDPR); ribonuclease UK114 were observed between the TAA and PLP groups. These proteins are involved in oxidative stress, heme and iron metabolism, cysteine metabolism, and branched-chain amino acid catabolism.

Conclusion: The proteomics data indicate that *P. linteus* may be protective against TAA-induced liver fibrosis via regulation of oxidative stress pathways, heat shock pathways, and metabolic pathways for amino acids and nucleic acids.

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Background

Most chronic liver diseases, including viral hepatitis (hepatitis B virus and hepatitis C virus), alcoholic liver disease, and biliary diseases [1], ultimately lead to liver fibrosis. Without effective treatments at an early stage, reversible liver fibrosis will lead to irreversible cirrhosis [2]. Oxidative stress may cause liver damage [3,4], and reducing oxidative stress by supplementation with antioxidants is effective for preventing liver fibrogenesis [5]. However, evidence for the efficacy of antioxidants, such as vitamin E and superoxide dismutase, in the treatment of human liver fibrosis has not been established [6].

Phellinus linteus (Berk. et Curt.) *Teng*, an orange-colored mushroom, belongs to the Hymenochaetaceae Basidiomycetes and has been considered useful in preventing and treating liver fibrosis and liver cancers owing to its strong anti-inflammatory, antioxidative, antiangiogenic, and anticancer properties [7-10]. *P. linteus* has been used in Chinese medicine for the treatment of tumors, menstrual irregularities, and liver-related illnesses [11]. Several reports from Korea and Japan have demonstrated that intake of *P. linteus* for a long time may induce spontaneous regression of hepatocellular carcinoma in patients with multiple metastases [12,13]. Some *in vivo* and *in vitro* studies have also demonstrated that *P. linteus* exerts antitumor effects on hepatocellular carcinoma [14-16].

Over the last decade, accumulating evidence suggests that *P. linteus* may protect the liver against fibrosis via its antioxidative property. A study in 2002 demonstrated that an extract of *P. linteus* was able to suppress carbon tetrachloride-induced late liver fibrosis by reducing peroxidation products, restoring the activities of catalase and superoxide dismutase, and reviving the expression of aerobic respiration enzymes [11]. Shon *et al.* [11] demonstrated that a *P. linteus* polysaccharide (PLP) fraction was able to inhibit cytochrome P450 isozymes in the liver. Furthermore, a retinoic acid derivative isolated from *P. linteus* was reported to decrease transforming growth factor-beta-induced early liver fibrosis by down-regulating reactive oxygen species generation and suppressing the expression of several proteins [11].

Although antioxidation is an important mechanism by which *P. linteus* suppresses liver fibrosis, the molecular mechanism of the antioxidative effect of *P. linteus* is still unclear. To date, studies on *P. linteus*-mediated protection of the liver against injury have only found a few target molecules [17]. With the development of proteomics technology, it is possible to cover the expression of more proteins acting within a biological context to investigate the cellular processes involved in disease pathogenesis with high-throughput and in a quantitative manner [18,19].

In the present study, we aim to assess the hepatoprotective effects of *P. linteus* against thioacetamide (TAA)-induced liver fibrosis by high-resolution two-dimensional polyacrylamide gel electrophoresis (2-DE) coupled with mass spectrometry technology.

Methods

Preparation of PLP

Sang Hwang 125 capsules containing a lyophilized hot water extract of wild-type *P. linteus* were donated by Dr. Frankie Chan (Amazing Grace Health Products Limited Partnership, Thailand). Each Sang Hwang capsule contained 400 mg of pure extracts from natural *P. linteus*. The polysaccharides and glucan contents of the *P. linteus* natural compound are 53–63% and 24%, respectively, as previously reported by us [20]. This natural compound has been shown to possess strong antioxidative and immunomodulatory properties [21]. PLP was prepared by dissolving 100 g of freeze-dried powder from Sang Hwang 125 capsules in 1 L of distilled water, followed by the addition of 2 L of ethanol (Merck, Germany) at −20°C. The precipitated polysaccharides were collected by centrifugation at 3000 × g for 1 h, dissolved in a small volume of distilled water, and lyophilized. The resulting powder was stored at −20°C until use.

Animal experiments

Eight-week-old male Sprague–Dawley rats (weighing approximately 200 g) were obtained from Hallym University (Korea). All rats were kept in an animal house under a 12-h/12-h light/dark cycle, with controlled temperature and humidity and free access to food and water. After 1 week of acclimatization, the rats were arbitrarily divided into three groups: Normal group; TAA group, in which rats received TAA only; and PLP group, in which rats received PLP and TAA. TAA (Sigma-Aldrich, USA) was intraperitoneally injected at a dose of 200 mg/kg body weight twice a week for 4 weeks. PLP was given orally at a dose of 50 mg/kg body weight twice a day from the beginning of the TAA treatment until the end of the experiment. All rats were euthanized after 4 weeks by intraperitoneal injection of 200 mg/kg sodium pentobarbital (Sigma-Aldrich, USA). Dissection was carried out, and liver samples were fixed in 10% buffered formalin solution (Surgipath, Germany) for histological staining. Tissues from the same portion of the liver were collected from the TAA and PLP groups for the proteomics analysis.

The study protocol was approved by the Hallym University, South Korea. Animal care complied with institutional guidelines.

Histological examination of the liver

The fixed liver tissues were embedded in paraffin and sectioned at 5-μm thickness. For each liver sample, the stage of hepatic fibrosis was established. The liver

sections were stained with Masson's trichrome (Sigma-Aldrich, USA) and observed under NIKON model SE microscope (NIKON, Japan) to evaluate the degree of fibrosis.

Sample preparation for proteomics analysis

Liver samples were snap-frozen in liquid nitrogen and stored at −80°C for the proteomics analysis. The frozen liver tissue samples from the TAA and PLP groups were disrupted with a tissue teaser (Biospec Products, USA) in a lysis buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA disodium salt, 1 mM dithiothreitol (DTT) (USB, USA), 1% (v/v) Triton X-100 (USB, USA), and 1% (v/v) Protease Inhibitor Cocktail Set III (Bio-Rad, USA). The superfluous salt in the extract was removed by incubation with 20% (w/v) trichloroacetic acid (TCA)-acetone solution and 20 mM DTT in acetone (Merck, Germany) for 4 hours at −40°C. The protein pellet was obtained by centrifugation at 15,800 × *g* for 30 min at 4°C. Excess TCA was removed by three washes with acetone containing 20 mM DTT. After air-drying, the protein pellet was resuspended in buffer comprising 7 M urea, 2 M thiourea, 100 mM DTT, 5% (v/v) glycerol, and 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (USB, USA), and the resulting protein solution was stored at −80°C until 2-DE analysis. The protein concentration was determined by the Bradford assay (Bio-Rad, USA).

Two-dimensional gel electrophoresis

The 2-DE procedures were performed according to our previous study [22] with some modifications. The tissue samples were processed in duplicate and a total of 12 gels (six for the TAA group and six for the PLP group) were used. For the first-dimension electrophoresis, 100-μg protein samples were mixed with 350 μL of rehydration buffer comprising 9.5 M urea, 2% (w/v) CHAPS, 0.28% (w/v) DTT, 0.002% (w/v) bromophenol blue (USB, USA) and 1% (v/v) immobilized pH gradient buffer (pH 3–10) (Bio-Rad, USA), and then applied to an Ettan IPGphor 3 isoelectric focusing electrophoresis system (GE healthcare, USA). The samples were rehydrated for 7 h before isoelectric focusing with the following programs: (a) linear increase up to 500 V over 1 h; (b) holding at 500 V for 2 h; (c) linear increase up to 10,000 V over 4 h; (d) linear increase up to 10,000 V over 3 h; and (e) final hold at 10,000 V to reach a total of 120,000 V × h. The focused immobilized pH gradient gel strips were equilibrated for 15 min in a solution comprising 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS) and 20 mM DTT, followed by incubation with the same buffer containing 20 mM iodoacetamide (Sigma-Aldrich, USA) for another 15 min. The second-dimension separation was performed by 12.5% SDS polyacrylamide gel electrophoresis (PAGE) at a constant current of 30 mA for 30 min, followed by a 60-mA current for the rest of the analysis until the bromophenol blue line reach the bottom of the gels.

Image acquisition and analysis

After the 2-DE, the gels were stained with SYPRO® Ruby Protein Stain (Bio-Rad, USA) according to the manufacturer's protocol. The stained gels were scanned with a Molecular Imager PharosFX Plus System (Bio-Rad, USA) and analyzed by PDQuest 8.0 software (Bio-Rad, USA). Each expression level was calculated as the percentage volume (% vol), and exported for statistical analysis. The relative intensities of spots were used for comparison between the two groups, and only those spots with significant differences (≥ 1.5-fold increase or decrease; $P < 0.05$) were selected for protein identification.

Protein identification

Spots showing differential expression ($P < 0.05$) between the TAA and PLP groups were sent to the Genome Research Centre (The University of Hong Kong, Hong Kong) for protein identification. The proteins were digested with sequencing grade modified trypsin (Promega, USA) and applied to matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer analysis using a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, USA). Matches between the experimental data and mass values calculated from a candidate protein were carried out by Mascot search engine (Matrix Science, UK) that uses MS data to identify proteins from the NCBInr database with taxonomy limited to *Rattus norvegicus*. The database allowed up to one missed cleavage and the mass tolerance was set as 75 ppm peptide limited by fixed modification of carbamidomethyl and variable modification of oxidation, with monoisotopic values. Mascot reported the molecular weight search (MOWSE) score, which is calculated by −10 × log10 (P), where P is the probability that the observed match is a random event. The P value is limited by the size of the sequence database being searched (limited by taxonomy), the conditions, and the settings of trypsin digestion. Each calculated value that falls within a given mass tolerance of an experimental value counts as a match. The accepted threshold is that an event is significant if it would be expected to occur at random with a frequency of < 5%. In this study, a protein match with a score of > 71 was regarded as significant.

Western blot analysis for validation of differentially expressed proteins

Western blot analysis was employed to validate the proteomic data. Liver protein extracts were mixed with sample buffer (62.5 mM Tris–HCl, pH 6.8, 25% (v/v)

glycerol, 2% (w/v) SDS, 350 mM DTT, and 0.01% (w/v) bromophenol blue) at a ratio of 1:1 and incubated in boiling water for 5 min. Aliquots of the samples (30 mg of protein) were separated by electrophoresis in 12.5% SDS-PAGE gels at constant voltage (120 V) and then transferred to polyvinylidene difluoride membranes (GE Healthcare, USA) using a TE77 PWR Semi-dry Transfer Unit (GE Healthcare, USA). The membranes were blocked with 5% (w/v) non-fat dry milk in phosphate buffer saline overnight at 4°C. The membrane was incubated with primary antibodies: anti-haptoglobin (1:1000), anti-hemopexin (1:1000;), anti-hemoglobin (1:1000), anti-GSTA4 (1:500), and anti-GSTmu (1:1000) (Abcam, USA) for one hour and then incubated with their corresponding secondary horseradish peroxidase-conjugated antibodies (Bio-Rad, USA) for another one hour. The blots were washed five times with 0.05% Tween-20 in phosphate buffer saline between steps. Proteins were detected with an enhanced chemiluminescence system (GE Healthcare, USA) and the band intensity was measured with the Quantity One software (Bio-Rad, USA).

Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was employed to verify the differentially expressed proteins identified by the proteomics analysis. Total RNA was extracted from liver samples in the TAA and PLP groups using TRIzol® (Invitrogen, USA). Aliquots of the total RNA (5 μg) were reverse-transcribed with Super Script III (Invitrogen, USA) in the presence of oligodeoxythymidylic acid primers (Sigma-Aldrich, USA) according to the manufacturer's instructions. PCR was performed with an iCycler Thermal Cycler (Bio-Rad, USA). cDNA (0.5 μL) were used for each PCR amplification in a total reaction volume of 15 μL using iQ SYBR Green Super Mix (Bio-Rad, USA), and all reactions were performed in duplicate. A total of 11 genes were examined, including ribonuclease UK114, hemopexin, preprohaptoglobin, glutathione S-transferase alpha-4 (*Gsta4*), branched chain keto acid dehydrogenase heterotetrameric E1 subunit alpha (*Bckdha*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), haptoglobin, thiosulfate sulfurtransferase (*Tft*), betaine-homocysteine S-methyltransferase 1 (*Bhmt1*), quinoid dihydropteridine reductase (*Qdpr*), and dihydrofolate reductase (*Dhfr*) because they showed significantly different expression levels in the 2-DE proteomics analysis, plus ubiquitin C as an internal control. The primers in Table 1 were used for the PCR with mentioned annealing temperature. The amplification was initiated by 4 min

Table 1 Primers used for the PCR analyses

Gene	Primer sequence (5'-3')	Fragment size (bp)	Annealing temperature (°C)
Preprohaptoglobin	F[1]: TGCCTATCTGCCTGCCTTC	316	58
	R[2]: GTGTCCTCCTCCGTGTCAT		
Hemopexin	F: AAGCCAGACTCAGATGTAA	479	55
	R: AAGCAGTAGTAGCGTTCA		
Gsta4	F: GGACCTGATGATGATGATTATC	446	54
	R: TATCTTGCCTCTGGAATGC		
Bckdha	F: AGCGTCACTTCGTCACCATT	547	60
	R: GCCTTCTCCTGTTCCTCATCC		
Bhmt	F: CAGACACCTTCCTACCTCAG	281	52
	R: CAGTTCACACCGACAATGG		
Dhfr	F: CTTGACGGCACTCTAAGC	304	52
	R: CTCCTTGTGGTGGTTCCT		
Qdpr	F: GATGTGGTGGAGAATGAAGAGG	241	56
	R: AGTGGCTAGAGATGGTGGATG		
Gapdh	F: CATGACCACAGTCCATGCCATC	451	60
	R: CACCCTGTTGCTGTAGCCATATTC		
Uk114	F: GCATGTCGTCAATAATCAGA	443	54
	R: CTCCAGAGTCAGCATCAG		
Tft	F: GGTTCATCAGGTGCTCTATCG	311	58
	R: CCAGGTCGTCTCCATCGTATA		
Ubiquitin C	F: TGGAGGTCGAGCCCAGTGTTA	105	58
	R: CCCAAGAACAAGCACAAGAAGGGCT		

[1]F: Forword.
[2]R: Reverse.

denaturation at 94°C for 1 cycle, followed by 30 cycles at 94°C for 30 s, specially annealing temperature of each gene for 30 s, and 72°C for 1 min using a Bio-Rad Icycler PCR thermocycler 96 well thermal thermo cycler (Bio-Rad, USA). After the last cycle of amplification, samples were incubated for 7 min at 72°C. The PCR products were examined in 1% agarose gels stained with 0.01% SYBR® Safe DNA Gel Stain (Invitrogen, USA) and analyzed using Quantity One software (Bio-Rad, USA).

Statistical analysis

All data are presented as the mean ± standard deviation (SD). The significance of differences in data between the groups was determined by one-way analysis of variance followed by the Tukey test for equality of variances using SPSS 17.0 (IBM, USA). Differences were considered statistically significant at $P < 0.05$.

Results

Histological assessment of liver fibrosis

TAA treatment of rats for 4 weeks resulted in liver fibrosis, which was characterized by alterations in the quality of the hepatic extracellular matrix (Figure 1B&C), compared with the livers of rats in the Normal group (Figure 1A). Extended collagen deposition and large septa of the hepatic lobules were observed after 4 weeks of TAA treatment (Figure 1B). In addition, lymphoid infiltration was observed around the central and portal veins in the TAA-treated livers. PLP treatment markedly reduced the severity of the fibrosis and inflammation induced by TAA (Figure 1C).

Identification of protein spots on 2-DE gels

On each 2-DE gel, nearly 1000 individual protein spots were detected, and 13 spots with notable changes found by the PDQuest software between the PLP and TAA groups were identified by MS (Figure 2, Table 2). The proteins with increased expression levels in the PLP group compared with the TAA group included actin cytoplasmic 2, tubulin alpha-1C chain, galectin-5, BCKDHA, DHFR, preprohaptoglobin, GSTA4, QDPR, GAPDH, and TFT. The proteins with decreased expression levels in the PLP group compared with the TAA group were hemopexin, ribonuclease UK114, and BHMT1.

Western blot analysis for validation of differentially expressed proteins in the proteomics analysis

Owning to the limitations of anti-rat protein antibodies, many of the identified differentially expressed proteins could not be measured by western blot analysis. Haptoglobin, hemopexin, heat-shock protein 70 (HSP70), and GSTA4 were successfully measured and used to validate the results obtained in the proteomic analysis. The western blot results were in general agreement with the differentially expressed proteins obtained in the proteomic analysis. As shown in Figure 3, the level of hemopexin ($P = 0.049$) was lower and the levels of haptoglobin ($P = 0.042$) and GSTA4 ($P = 0.040$) were much higher in the PLP group compared with the TAA group. The levels of hemoglobin ($P = 0.047$) and HSP70 ($P = 0.041$) were higher in the PLP group than in the TAA group. GSTmu did not show a significant difference in the western blot analysis.

Quantitative RT-PCR for gene expression analysis

To investigate whether the expression changes of the identified proteins occurred at the transcriptional level, we determined the mRNA expression changes of these proteins by semiquantitative RT-PCR. As shown in Figure 4, the mRNA expression of many of the identified genes changed in a similar tendency as their protein expression change showed in 2D proteomic results, suggesting that the effects of *P. linteus* were exerted at the protein expression level, *i.e.* focused on the translation and post-translation steps. Haptoglobin, BCKDHA, and BHMT showed significant differences between the TAA and PLP groups.

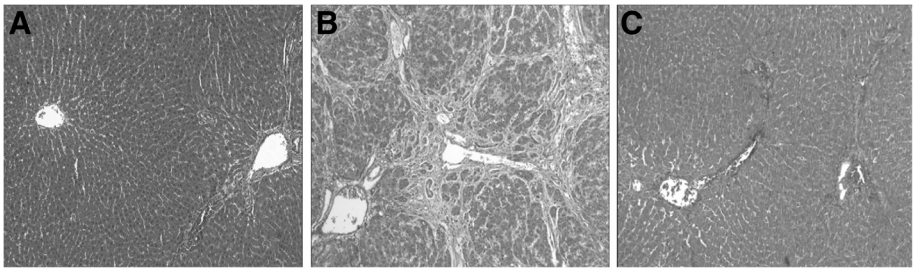

Figure 1 Photomicrographs of rat livers. The livers were sectioned at 5-μm thickness and the sections were stained with Masson's trichrome. **A**: Normal group. **B**: TAA group. **C**: PLP group. Extended collagen deposition and large septa of the hepatic lobules are observed in the TAA-treated liver (**B**) compared with the normal liver (**A**). In addition, lymphoid infiltration is observed around the central and portal veins in the TAA-treated liver. PLP treatment markedly reduces the severity of the fibrosis and inflammation induced by TAA (**C**).

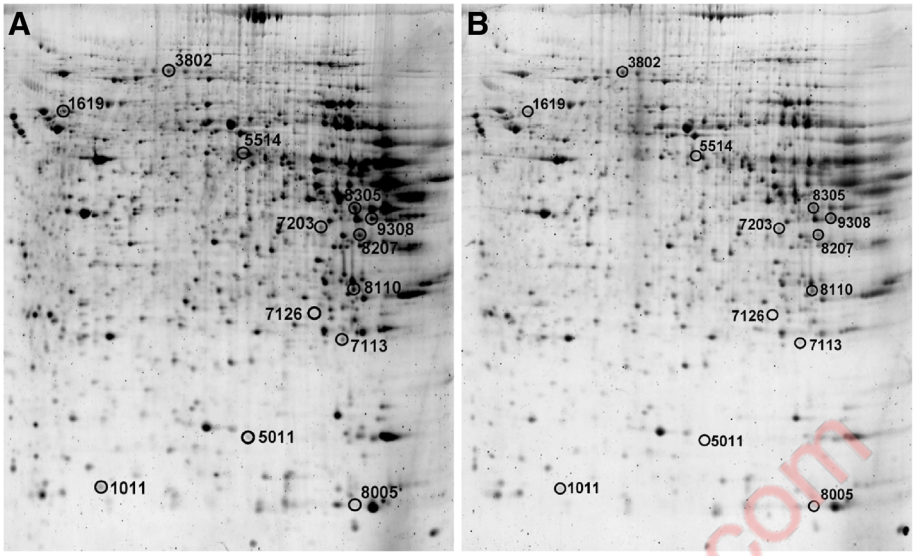

Figure 2 Representative 2-DE gel maps of the liver proteomes of rats in the TAA (A) and PLP (B) groups.

Discussion

The present study demonstrates that a natural product derived from *P. linteus* was able to protect against liver fibrosis induced in rats by chronic insult with TAA. The histopathological data clearly showed a reduction in collagen accumulation in the liver with PLP treatment. The present study thus supports the earlier findings that *P. linteus* possesses the capability to suppress liver injury [17,23] and exhibits strong and specific inhibitory activities to reduce peroxidation products and increase antioxidant enzymes in the liver [17,23].

By using a 2-DE gel proteomics approach, we identified 13 differentially expressed hepatic proteins in the TAA-induced liver fibrosis rats in response to PLP treatment. Of these, 10 proteins showed increased expression and three proteins showed reduced expression, and the expression changes ranged from ±1.5-fold to ±2.5-fold (Figure 5). When these proteins are categorized according to their biochemical and physiological functions, we found associations with oxidative responses, molecular chaperones, heme and iron metabolism, cysteine metabolism, branched-chain amino acid metabolism, energy

Table 2 Differentially expressed liver proteins between the TAA-induced liver fibrosis rats in the PLP and TAA groups

Spot No.[1]	Protein name	GenInfo identifier[2]	Protein score[3]	Expression quantity (×10^2) TAA	Expression quantity (×10^2) PLP	Expression change (PLP/TAA)	*P*	*pI*[4]	*Mr* (kDa)[4]
1011	actin, cytoplasmic 2	gi|4501887	65	62.6 ± 24.5	97.1 ± 13.0	1.6	0.012	5.31	42.1
1619	tubulin alpha-1C chain	gi|58865558	254	29.4 ± 13.0	55.4 ± 14.0	1.9	0.014	4.96	50.6
3802	hemopexin	gi|122065203	262	100.5 ± 34.1	61.4 ± 15.4	−1.6	0.028	7.58	52.0
5011	galectin-5	gi|785053	120	11.5 ± 9.5	75.3 ± 44.9	6.5	0.028	6.95	15.5
5514	Bckdha protein	gi|59808237	88	41.3 ± 9.1	61.0 ± 11.5	1.5	0.008	6.4	37.6
7113	dihydrofolate reductase	gi|18426814	100	49.3 ± 14.7	73.8 ± 14.0	1.5	0.015	6.77	21.7
7126	Glutathione S-transferase alpha-4	gi|157820217	67	15.1 ± 7.6	28.9 ± 11.3	1.9	0.033	6.77	25.6
7203	preprohaptoglobin	gi|204657	75	54.8 ± 20.6	92.5 ± 16.2	1.7	0.005	7.16	30.4
8005	ribonuclease UK114	gi|47168636	159	172.2 ± 69.2	78.9 ± 45.1	−2.2	0.020	7.79	14.4
8110	quinoid dihydropteridine reductase, isoform CRA_c	gi|149047263	135	83.7 ± 22.3	128.5 ± 25.5	1.5	0.009	9.69	27.9
8207	betaine-homocysteine S-methyltransferase 1	gi|13540663	142	128.6 ± 42.6	65.1 ± 12	−2.0	0.006	8.02	45.4
8305	glyceraldehyde-3-phosphate dehydrogenase	gi|8393418	130	141.6 ± 69.1	225.8 ± 44.3	1.6	0.035	8.14	36.1
9308	thiosulfate sulfurtransferase	gi|57528682	256	63.8 ± 15.7	99.7 ± 22.8	1.6	0.010	7.71	33.6

[1]Spot no.: automatically assigned by the PDQuest software.
[2]GenInfo identifier: sequence identification number assigned by GenBank.
[3]Protein score: generated by the MS identification system.
[4]*Mr* and *pI*: relative molecular mass (*Mr*) and isoelectric point (*pI*) generated by the MS system.

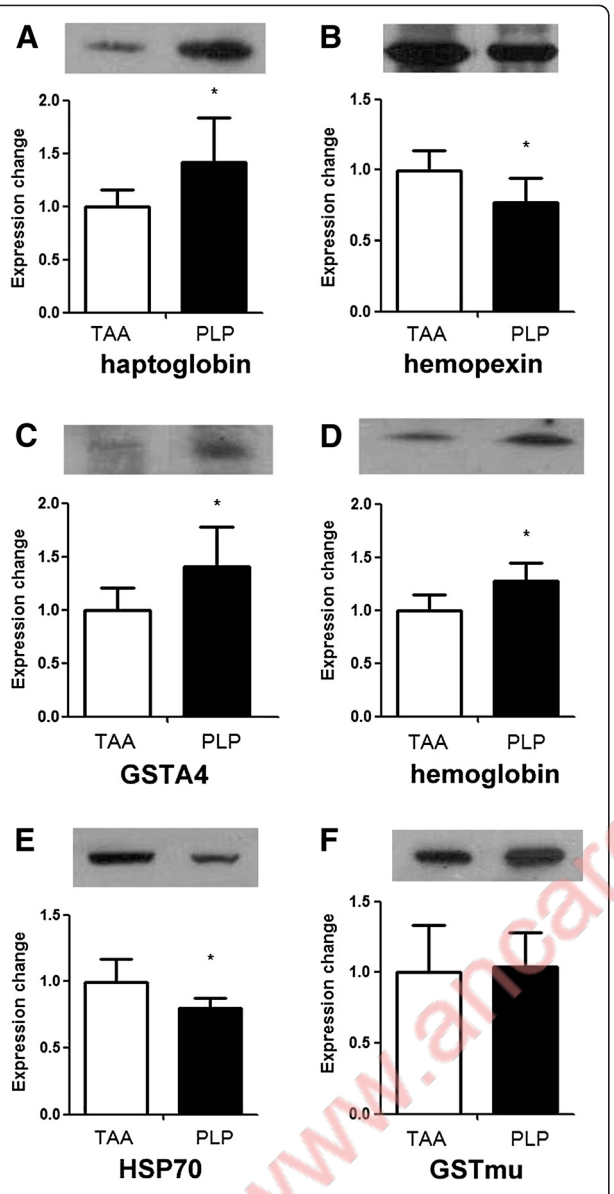

Figure 3 Western blot measurements of the haptoglobin, hemopexin, GSTA4, hemoglobin, HSP70, and GSTmu expression levels in the TAA-induced fibrotic livers with and without PLP treatment. The protein expression levels are presented as means ± SD ($N = 6$). *$P < 0.05$ *vs.* the TAA group.

metabolism, and glutathione metabolites (Table 3). Among these 13 proteins, the regulation of hemopexin, preprohaptoglobin, GSTA4, BHMT, BCKDHA, QDPR, DHFR, and galectin-5 expression could be important in the protective effects of *P. linteus* against liver fibrosis.

The proteomic data showed that the expression of preprohaptoglobin was 1.7-fold higher while that of hemopexin was 1.6-fold lower in the PLP group compared with the TAA group. The expression changes of these two proteins were validated by western blot analysis (Figure 3). The increase in hemopexin and decrease in haptoglobin are potential markers for fibrosis because of their involvement in the regulation of liver iron homeostasis [24]. The aspect of whether the protective effect of PLP against the TAA-induced liver fibrosis occurred *via* the regulation of iron homeostasis cannot be concluded in the present study, because the liver and serum iron concentrations were not determined. In a previous study, chelation of ferrous ions by *P. linteus* was described, and PLP was able to protect hepatocytes against iron overload-mediated oxidative stress [21]. Iron homeostasis regulation has been suggested as a potential PLP treatment target in liver fibrosis [25].

Glutathione (GSH) plays an important role in cellular detoxification, because it effectively scavenges free radicals and other reactive oxygen species. In GSH-related antioxidative detoxification, glutathione S-transferases (GSTs) play central role; GSTA4 plays a role in the cellular defense against oxidative stress and lipid oxidation during liver injury [26]. Dwivedi *et al.* [27] demonstrated that mGSTA4 null (−/−) mice showed much quicker and greater carbon tetrachloride-induced hepatotoxicity than wild-type (+/+) mice. In the present study, the expression of GSTA4 was 1.9-fold higher in the PLP group than in the TAA group, and the change was confirmed by western blot analysis. The upregulated expression of GSTA4 might protect the liver against the injury and oxidative stress induced by TAA. However, the western blot analysis did not show a significant change in GSTmu between the PLP and TAA groups. These results could arise through non-specificity of the antibody for GSTmu or because GSTA4 was likely to be regulated by *P. linteus.*

The expression of BHMT was 2-fold lower in the PLP group than in the TAA group, suggesting that homocysteine was inclined to be converted to cysteine in the transsulfuration reaction, generating more cysteine for GSH synthesis. *P. linteus* may promote the accumulation of substrates for GSH synthesis, cysteine and glutamate [28], by regulating the expression levels of BHMT and BCKDHA [29]. BHMT reduces the conversion of homocysteine to cysteine by catalyzing the remethylation of homocysteine back to methionine [30].

Branched-chain amino acid (BCAA) catabolism is an important intercellular source of glutamate [31]. The branched-chain α-keto acid dehydrogenase (BCKD) complex is the rate-limiting enzyme for the whole BCAA catabolism. The *Bckdha* gene encodes the E1 α subunit of the BCKD [32]. The expression of BCKDHA was 1.5-fold higher in the PLP group than in the TAA group, suggesting that more glutamate was generated for GSH synthesis in the PLP group.

Several proteins that showed higher expression in the PLP group are involved in amino acid metabolism and nucleic acid metabolism. These include BCKDHA (1.5-

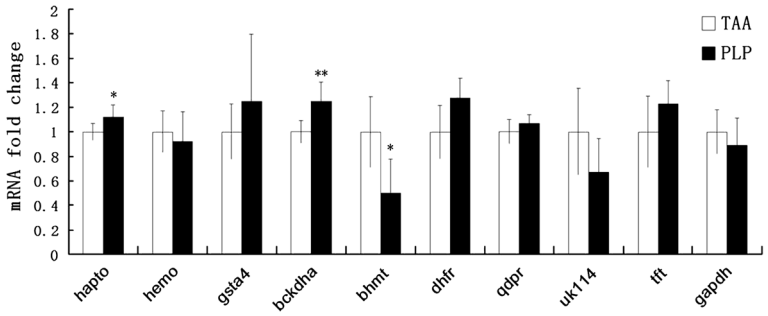

Figure 4 Semiquantitative RT-PCR analyses of the effects of PLP on the gene expression changes of significantly altered proteins during TAA-induced liver fibrosis. The mRNA expression levels are presented as means ± SD ($N = 6$). *$P < 0.05$,**$P < 0.01$ *vs.* the TAA group.

fold), QDPR (1.6-fold), and DHFR (1.5-fold). In clinical treatment of liver diseases, supplementation with BCAAs is considered useful to relieve protein malnutrition [33,34]. QDPR is an enzyme that takes part in the tetrahydrobiopterin recycling pathway, and tetrahydrobiopterin is the precursor of phenylalanine and tyrosine [35]. The higher expression of QDPR in the PLP group suggests that PLP may expedite protein and nucleic acid synthesis in the fibrotic liver. DHFR is important for regulating the cellular amount of tetrahydrofolate, which is essential for purine and thymidylate synthesis [36,37]. The higher expression of DHFR in the PLP group indicates that PLP may aid in the regeneration of liver injury. The expression of ribonuclease UK114, a translational inhibitor mostly present in the liver and kidney, was 2.2-fold lower in the PLP group, meeting the requirement for protein synthesis for liver regeneration. In a clinical study, downregulation of ribonuclease UK114 was observed in human hepatocellular carcinoma [38].

GAPDH catalyzes a step of glycolysis. The expression of GAPDH was 1.6-fold higher in the PLP group, suggesting a higher energy requirement for liver amelioration. Several studies have illustrated that GAPDH may work in non-metabolic processes, such as transcription regulation [39] and apoptosis initiation [40,41]. This may be another reason for the upregulation of GAPDH in the PLP group.

Galectins comprise a family of evolutionarily conserved glycan-binding proteins that take part in acute and chronic inflammation [42,43]. Galectin-5 contributes to erythrocyte differentiation and reticulocyte

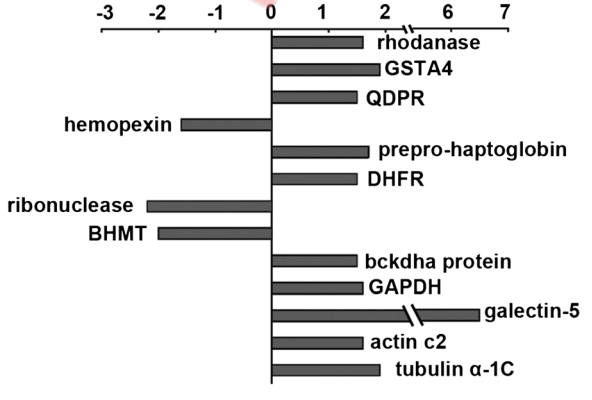

Figure 5 Effects of PLP on the expression changes of significantly altered proteins involved in TAA-induced liver fibrosis (PLP/TAA).

Table 3 Major biofunctions of the identified proteins

Protein name	Subcellular location	Major functions
Anti-oxidant effects		
hemopexin	Extracellular region	The highest binding affinity for heme, iron metabolism
preprohaptoglobin	Extracellular region	The highest binding affinity for hemoglobin
glutathione S-transferase alpha-4 (GSTA4)	Cytoplasm	GSH-related detoxification
betaine-homocysteine S-methyltransferase 1 (BHMT1)	Cytoplasm	cysteine metabolism and GSH synthesis regulation
Bckdha protein	Mitochondrion matrix	Branched-chain amino acids catabolism
Liver amelioration		
dihydrofolate reductase (DHFR)	Cytoplasm	Synthesis of nucleic acid precursors
quinoid dihydropteridine reductase (QDPR)	Cytoplasm. Synaptosome	Tetrahydrobiopterin recycle, amino acid metabolism
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Cytoplasm. Nucleus.	Glucose metabolism, initiation of apoptosis
ribonuclease UK114	Mitochondrion. Cytoplasm. Nucleus. Peroxisome	Translational inhibition
galectin-5	Cytoplasm Cell surface of rat reticulocytes and erythrocytes	Erythrocyte differentiation and reticulocyte maturation

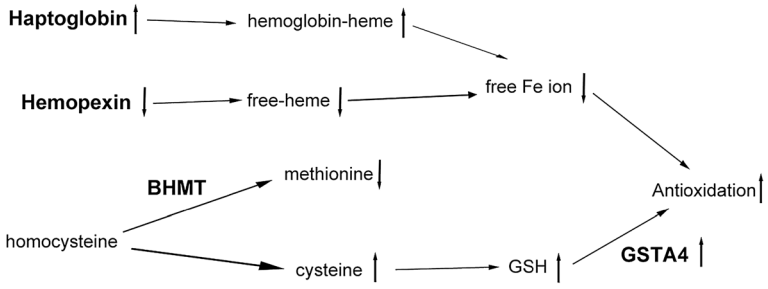

Figure 6 Proposed mechanistic pathways for the protective effect of PLP against TAA-induced liver fibrosis in rats. Liver proteins with significant expression changes detected by the 2-DE proteomics analysis are used to construct the possible pathways. These include the antioxidant system, iron metabolism regulation pathways (haptoglobin, hemopexin), and amino acid and nucleic acid metabolic pathways (homocysteine, BHMT, GSTA4). Upward arrowheads indicate upregulation or increasing, and downward arrowheads indicate downregulation or decreasing. BCAAs: branched-chain amino acids; BCKAs: branched-chain α-keto acids; BC acyl-CoA: branched-chain acyl-CoA.

maturation, but its function in liver injury remains unclear [44,45]. The much higher expression of galectin-5 in the PLP group suggests that PLP may promote erythropoiesis, inflammation regulation, and liver regeneration.

Based on the proteomics data, we propose that the antioxidant pathway, iron metabolism pathway, and metabolic regulation of amino acids and nucleic acids are a few key networks involved in the hepatoprotective effect of PLP against TAA (Figure 6). Our western blot analyses further indicated that the PLP-mediated protection against TAA-induced hepatic injury involves the heat shock pathway. HSP70 has a crucial cytoprotective function mediated by its function as a molecular chaperone. A high level of HSP70 is a stress marker for liver injury [46,47]. The aspect of whether the reduced level of HSP70 represented a less inflammatory state of the TAA-treated liver with PLP treatment awaits confirmation by functional proteomics analyses in future studies.

Conclusion

The present study has demonstrated that PLP can protect rats against TAA-induced liver fibrosis in at least two possible ways: 1) protection of the liver against oxidative stress, especially by scavenging of iron-related free radicals; and 2) regulation of the metabolism of amino acids and nucleic acids for liver amelioration. Our findings provide novel molecular mechanisms for the protective effects of *P. linteus* against liver fibrosis.

Abbreviations

PLP: *Phellinus linteus* polysaccharide; TAA: Thioacetamide; 2-DE: Two-dimensional polyacrylamide gel electrophoresis; MALDI-TOF/TOF MS: Matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight mass spectrometry; RT-PCR: Reverse transcription polymerase chain reaction; GSTA4: Glutathione S-transferase alpha-4; BCKDHA: Branched chain keto acid dehydrogenase heterotetrameric E1 subunit alpha; GSTmu: Glutathione S-transferase mu; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TFT: Thiosulfate sulfurtransferase; BHMT1: Betaine-homocysteine S-methyltransferase 1; QDPR: Quinoid dihydropteridine reductase; DTT: Dithiothreitol; TCA: Trichloroacetic acid; CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; SDS: Sodium dodecyl sulfate; PAGE: Polyacrylamide gel electrophoresis; MOWSE: Molecular weight search; DHFR: Dihydrofolate reductase; HSP70: Heat shock protein 70; GSH: Glutathione; GSTs: Glutathione S-transferases; BCAA: Branched-chain amino acid; BCKD: Branched-chain α-keto acid dehydrogenase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JMFW conceived and designed the study. HLW performed experiments, data acquisition, and results interpretation. HJP designed and GW conducted the animal experiments. PPJ and WHS performed the proteomics analysis. LJLDvG coordinated the study. JMFW, HLW, and LJLDvG wrote the manuscript. All authors read and approved the final version of the manuscript.

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Anti-tumor effects of proteoglycan from *Phellinus linteus* by immunomodulating and inhibiting Reg IV/EGFR/Akt signaling pathway in colorectal carcinoma

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ABSTRACT

Proteoglycan (P1) purified from *Phellinus linteus* has been reported to have anti-disease activities. The objectives of our research were to determine the anti-tumor effect and possible mechanisms of P1 on human cancer cells. Cell inhibition assay showed that P1 has an antiproliferative effect on HepG2, HT-29, NCI-H 460 and MCF-7 human colon cancer cells, especially it was very effective in inhibiting HT-29 cells. When HT-29-bearing mice were treated with P1(100 mg/kg), there was relative increase in spleen and thymus weights, the plasmatic pIgR and IgA levels were significantly increased, also there was a notable decrease in plasmatic PGE2, Reg IV, EGFR and Akt concentrations measured by ELISA. RT-PCR analysis suggested that P1-induced HT-29 apoptosis appeared to be associated with a decrease in the levels of expression of Reg IV and EGFR. These results suggest that P1 might have two potential roles in treating cancer; it acts as an immunopotentiator partly through protecting T cells from escaping PGE2 attack and enhancing the mucosal IgA response, and as a direct inhibitor by disrupting the Reg IV/EGFR/Akt signaling pathway.

1. Introduction

Phellinus linteus (PL), is a kind of mushroom that grows mainly on wild mulberry tree trunks, has been used as a traditional medicine in China, Korea, Japan and other Asian countries for treatment of various diseases, including oral ulcer, gastroenteric disorder, lymphatic disease and various cancers. Today, PL draws greater attention by more and more people because of its anti-tumor properties. Polysaccharides and proteoglycans extracted from PL are thought to be the active ingredients involved in anti-tumor effect [1]. To investigate the potential anticancer mechanisms of these polysaccharides, a large number of studies have focused on the functional activations of immune cells. It has been reported that polysaccharides and proteoglycans extracted from PL could stimulate the proliferation of T lymphocytes and the humoral immune function, including acting as a polyclonal activator on B cells, thereby inducing the secretory and cellular macrophage response [2,3]. In recent years, polysaccharides isolated from PL have also been shown direct antitumor effects through apoptosis and blocking cell cycle progression, adhesion and invasion of human colon cancer cells [3,4]. These results suggested that the effects of these polysaccharides not only are the immunomodulatory activity, but also could directly act on the tumor cells.

Although the antitumor effect of PL extract is apparent, the underlying mechanism(s) is still not clear. In this study, to explore the underlying mechanism(s) of the anticancer effects for this famous traditional medicine, a novel heteropolysaccharide named 'P1' was isolated from PL by HPLC isolation method and its effect was studied. Further experiments demonstrated that P1 could inhibit cellular proliferation of HT-29 cells by down regulating the expression of Reg IV and EGFR genes, and up-regulating pIgR mRNA level. In HT-29-bearing model mice, the significant changes of plasma biochemical parameters show that P1 inhibited colorectal carcinoma by not only by enhancing the immune response of T cells and IgA, but disrupting the Reg IV/EGFR/Akt signaling pathway as well.

2. Materials and methods

2.1. Preparation of the polysaccharide (P1)

Fresh-fruiting bodies of *P. linteus* (PL) were collected from the mulberry trees on the Tong-lu Mountain in Zhejiang province of China, authenticated by Lin Hong, Department of Botany, Zhejiang Academy of Agricultural Science. The dried mushroom powder was defatted with ethanol and ethyl acetate three times at room temperature. Then it was extracted three times with boiling water for 2 h, concentrated and treated with three volumes of ethanol. The

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deposit collected was redissolved in distilled water and further purified using HPLC (Waters, USA). Its purity was more than 95% based on HPLC analysis (column: Ultrahydrogel™ Linear, mobile phase: water; flow rate: 0.6 ml/min; detection: 2414, and temperature: 50 °C). The purified solution of polysaccharide (P1) was collected, concentrated and lyophilized to dryness by low pressure evaporation at −50 °C.

2.2. Analysis of polysaccharide and protein contents

The protein content was measured by Lowry's method using bovine serum albumin as the standard protein [5]. The average molecular weight was determined by HPLC using a Waters 600 HPLC system, a Model 600 pump, a Waters 2410 RI detector, a Waters 2487 dual wavelength absorbance detector and an on-line de-gasser. In order to analyse the monosaccharide compositions, P1 was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 °C for 2 h, and the monosaccharides were identified by high-performance anion-exchange chromatography (HPAEC) using a Dionex LC30 equipped with a CarboPac™PA20 column (3 mm × 150 mm) [6,7].

2.3. Cell culture media and supplements

HepG2 (hepatocellular carcinoma), HT-29 (colon carcinoma cell), NCI-H460 (lung cancer cell), MCF7 (breast cancer cell) and L-929 (mouse fibroblast cell) cells were obtained from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences and grown in RPMI 1640 containing 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. P1 was dissolved with 0.1% DMSO and adjusted to final concentrations with culture medium before use. RPMI 1640 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). RevertAid™ First-Strand cDNA Synthesis Kits were purchased from Fermentas (Lithuania). Cisplatin (CDDP) was purchased from Kunming Gui-Yan Pharmaceutical Co. Ltd. All other chemicals were of the highest purity available.

2.4. Cell inhibition assay

The inhibitory effects of P1 on the cells (HepG2, HT-29, NCI-H 460, MCF-7 and L-929) were evaluated by the analysis of viable cells number determined with a MTT-based colorimetric assay [8]. Briefly, the cells were cultured in RPMI 1640 complete medium. Sterilized test samples were added to a 96-well plate, containing all tested cells (1×10^5 cells/well) to give a final concentration of P1 (15.625, 31.25, 62.5, 125, and 250 μg/ml), DMSO was used as negative control, and CDDP was used as a positive control. After cultivation for 48 h at 37 °C in a humidified 5% CO_2 incubator, the percentage of viable cells was determined by MTT assay, reading absorbance at 570 nm with a Benchmark microplate reader (Bio-Rad, California). The inhibitory rates of cells were calculated by the following formula: %Inhibitory rate = 1 − (mean absorbency in test wells)/(mean absorbency in control wells) × 100%.

2.5. HT-29 cell proliferation assay

Cells were plated at a density of 1×10^5 cells/well on 24-well plates with various final concentrations of P1 (16.125 and 32.25 μg/ml) and cultured up to 96 h. At the indicated time points after the treatment with P1, the number of viable cells was determined by trypan blue exclusion at 24, 48, 72, and 96 h.

Table 1
Primers used in quantitative real-time reverse transcription-PCR.

Primer	Sequence 5′–3′	PCR product size (bp)
DDX32-F	AGCAAACACAAGGTGCAGCA	106
DDX32-R	AGACCGTCCTGTGGATGTGAA	
MCM2-F	TGTGATCGAAGACGACGTCAA	101
MCM2-R	CAAAAGTCTTGCGCATGCTG	
PIgR-F	AGCCGTCTATGTGGCAGTTGA	110
PIgR-R	CCCGAAAACCAGAGTCTAGCA	
RegIV-F	GCCAACACTTCCTGTGCAAGT	105
RegIV-R	GCAGATTTAGCCAGGCTAGCA	
EGFR-F	ATAGACGCAGATAGTCGCCCA	106
EGFR-R	GCATTCTTTCATCCCCCTGA	
β-Actin – F	CCATCATGAAGTGTGACGTGG	102
β-Actin – R	TCTGCATCCTGTCGGCAAT	

Key-DDX32: DEAH (Asp-Glu-Ala-His) box polypeptide 32, MCM2: minichromosome maintenance protein 2, pIgR: polymeric immunoglobulin receptor, RegIV: regenerating gene IV, EGFR: epidermal growth factor receptor.
F: forward primer, R: reverse primer, bp: base pairs (length of nucleic acid sequence).

2.6. Quantitative real-time reverse transcription-PCR analysis

Cells were plated at a density of 1×10^6 cells/well on 24-well plates with final concentrations of P1 (16.125 and 32.25 μg/ml) and cultured up to 48 h. At the indicated time points after the treatment, total RNA from HT-29 cells was extracted using TRIZOL reagent according to the supplier's instruction. RNA was quantitated by optical density measurement at 260 and 280 nm using a spectrophotometer, and integrity was confirmed by running 4 μl RNA on a 1.2% agarose gel.

The PCR primers of MCM2 (minichromosome maintenance protein 2), DDX32 (DEAH (Asp-Glu-Ala-His) box polypeptide 32), pIgR (polymeric immunoglobulin receptor), Reg IV (regenerating gene IV), EGFR (epidermal growth factor receptor) and β-actin were synthesized by Shanghai Shenergy Biocolor BioScience and Technology. The sequences of the primers used in this study are shown in Table 1. Reverse-transcription (RT) was performed from total cellular RNA using RevertAid™ First-Strand cDNA Synthesis Kit for RT-PCR, and carried out according to the method of Li et al. [9].

2.7. In vivo anti-tumor activity test and PGE2, pIgR, IgA, Reg IV, EGFR and Akt levels in plasma

Female BALB/c-nu/nu mice (6 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. All mice were housed under controlled conditions (a 12 h light/12 h dark cycle, 60% humidity and 25 ± 1 °C) with free access to standard pellet diet and water. 50 mice were divided into 5 groups. Except the normal control group, 4 groups were inoculated 0.2 ml of HT-29 cells suspension (2.5×10^7 cells/ml) at the nape subcutaneously to establish the colorectal carcinoma model mice at day 0. Test sample P1 (50 and 100 mg/kg), and 0.2 ml saline (as negative control) were administered intragastrically everyday, CDDP (2 mg/kg, as positive control) was injected into peritoneal cavity every other day. After the tumor inoculation, the maximal (a) and minimum (b) diameters of solid tumors were measured with vernier caliper every week, and the tumors volume calculated as $V = a \times b^2/2$. Compared with the control group, the anti-tumor activity of the tested samples was expressed as an inhibition ratio (percent) calculated as $((A - B)/A) \times 100\%$, where A and B are the average tumor volume of the control and treated groups. To determine the potential toxicity of P1, the body weights of mice were determined every week. At the end of experiment, plasma were collected from the tumor-bearing mice and measured using murine enzyme-linked immunosorbent assay (ELISA) kits (Purchased from R&D Systems (USA)) for PGE2, pIgR, IgA, Reg IV, EGFR and Akt according to the manufacturer's instruction. Spleen and thymus were excised from the animal and

weighed immediately. The spleen/thymus index was expressed as the spleen (thymus) weight (mg) relative to body weight (g). All animals used in the current study were handled and treated in accordance with guidelines of National Institutes of Health.

2.8. Statistical analysis

Results are reported as means ± S.D. ANOVA was used to evaluate the difference between multiple groups. If significance was observed between groups, a Duncan's multiple range test (DMRT) was used to test the means of two specific groups by a commercially available statistics software package (SPSS for Windows, V. 12.0, Chicago, USA), with $P < 0.05$ considered as significant.

3. Results

3.1. Investigation of the proteoglycan

The P1 was isolated from precipitated polysaccharide (crude proteoglycan fraction) of the *P. linteus*. P1 appeared as a symmetrical peak on HPLC and the average molecular weight was about 18.8 kDa. The monosaccharide analysis showed that it was composed of L-fucose, D-rhamnose, D-galactose, D-glucose, D-xylose and D-mannose, and a novel monosaccharide 3-O-Me-D-galactose was detected, which is similar to the report by Yang [6]. The corresponding molar ratios for the monosaccharides above were approximately 1:3.12:33.51:2.03:4.03:1.09:2.87 and the protein content was 8.45% in the extraction.

3.2. Inhibitory effects of P1on cancer cells and its cytotoxicity to normal cells

The inhibitory effects of P1 were tested on HepG2, HT-29, NCI-H 460 and MCF-7 human colon cancer cells under different dosages of 15.625, 31.25, 62.5, 125, and 250 μg/ml in vitro. As shown in Fig. 1A, a significant inhibition of P1 in all the cancer cells were observed at high dose of 250 μg/ml and the highest inhibition ratios were 88.91%, 92.79%, 79.71% and 73.99%, respectively. Meantime, P1 also exhibited a significant cytotoxicity against normal cells (L-929), the inhibition ratio was as high as 83.72%. However, when the dose was under 62.5 μg/ml, cytotoxicity to the normal cells (L-929) was not significant (inhibition ratio fewer than 10.0%). But no significant inhibitory activity was also observed to cancer cells at the low doses (under 62.5 μg/ml) except for HT-29 cells. The standard reference drug (CDDP) exhibited a significant dose-dependent inhibition of cell proliferation in all cell lines including the normal cells (L-929) (Fig. 1B). So the P1 may have a specific concentration range to treat colorectal carcinoma, and the proliferation of cells HT-29 was further investigated.

3.3. Effect of P1 on HT-29 cell proliferation

To explore the inhibitive effect and its mechanism(s) of P1 on HT-29 cell proliferation at non-toxic dose levels, P1 was added to the HT-29 cell culture medium maintained for 24, 48, 72, and 96 h, respectively. P1 treatments showed significant inhibition of HT-29 cell proliferation in the presence of 15.625 and 31.25 μg/ml (Fig. 2). The growth morphology of cells HT-29 treated with P1 for 48 h is shown in Fig. 3 (×100) after P1-treatment for 48 h. The control cells showed normal karyokinesis and proliferation as exponential growth (Fig. 3 A), the proliferations were inhibited and the number of the cells was lower evidently than control ($P < 0.01$) after treatment with P1 (15.625 and 31.25 μg/ml) and CDDP (0.625 μg/ml) (Fig. 3B–D). These results suggest that P1 may have a direct antitumor activity on HT-29 cells.

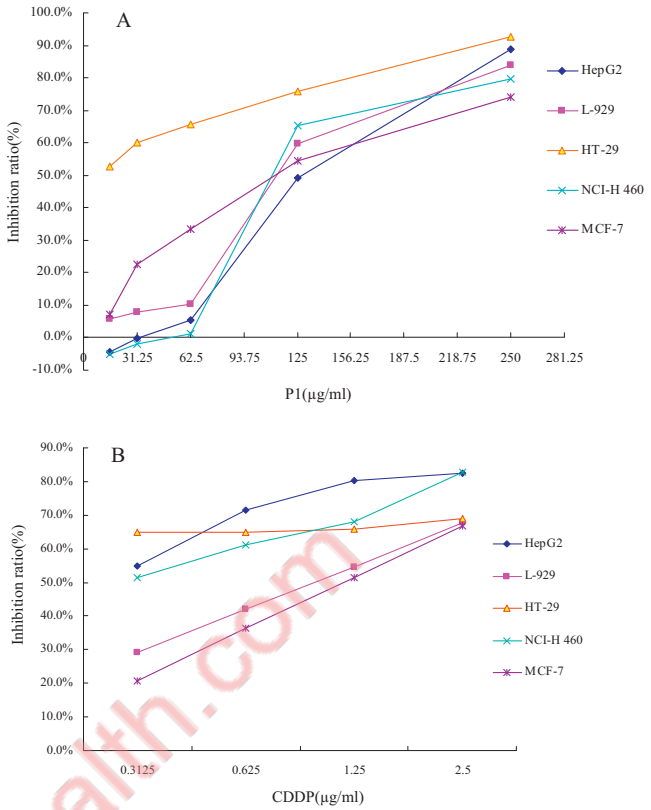

Fig. 1. Effect of P1 and CDDP on the proliferation of HepG2, HT-29, NCI-H 460, MCF-7 and L-929 cells. Cells were plated at a density of 1×10^5 cells/well on 96-well plates with various concentrations of P1 (15.625, 31.25, 62.5, 125, and 250 μg/ml) (A) and CDDP (0.3125, 0.625, 1.25, and 2.5 μg/ml) (B), and then cultured up to 48 h. After indicated time of culture, cell numbers were determined by MTT colorimetric assay. Data are mean ± S.D. of three repeated experiments.

3.4. Expression of MCM2, DDX32, pIgR, Reg IV and EGFR mRNA in HT-29 cells

Recent findings have demonstrated that many functional gene expression are frequently abnormal in various gastrointestinal tumors, in which the expression of MCM2 [10], DDX32 [11], Reg IV [12] and EGFR [13] are up-regulated, while pIgR is down-regulated in colorectal cancer cells, adenoma and carcinoma tissues [14]. In this study, to validate the inhibitory mechanism(s), the mRNA gene levels of MCM2, DDX32, pIgR, Reg IV and EGFR in the HT-29 cells were detected by quantitative real-time reverse transcription-PCR.

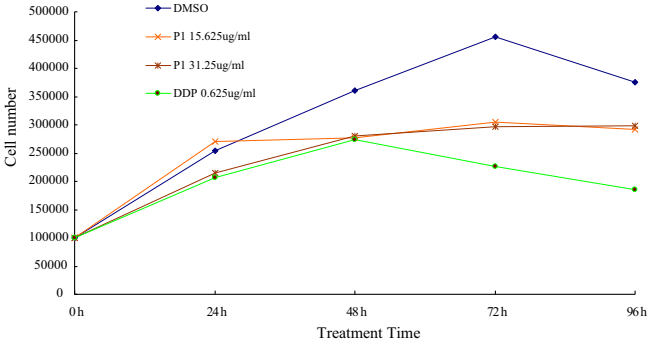

Fig. 2. The antiproliferative effect of P1 in HT29 cells. Cells were plated at a density of 1×10^5 cells/well on 24-well plates and treated with various concentrations of P1 (15.625 and 31.5 μg/ml) and CDDP (0.625 μg/ml) for 24, 48, 72 and 96 h. After treatment, cell viability was estimated by trypan blue dye exclusion method. Data are mean ± S.D. of three repeated experiments.

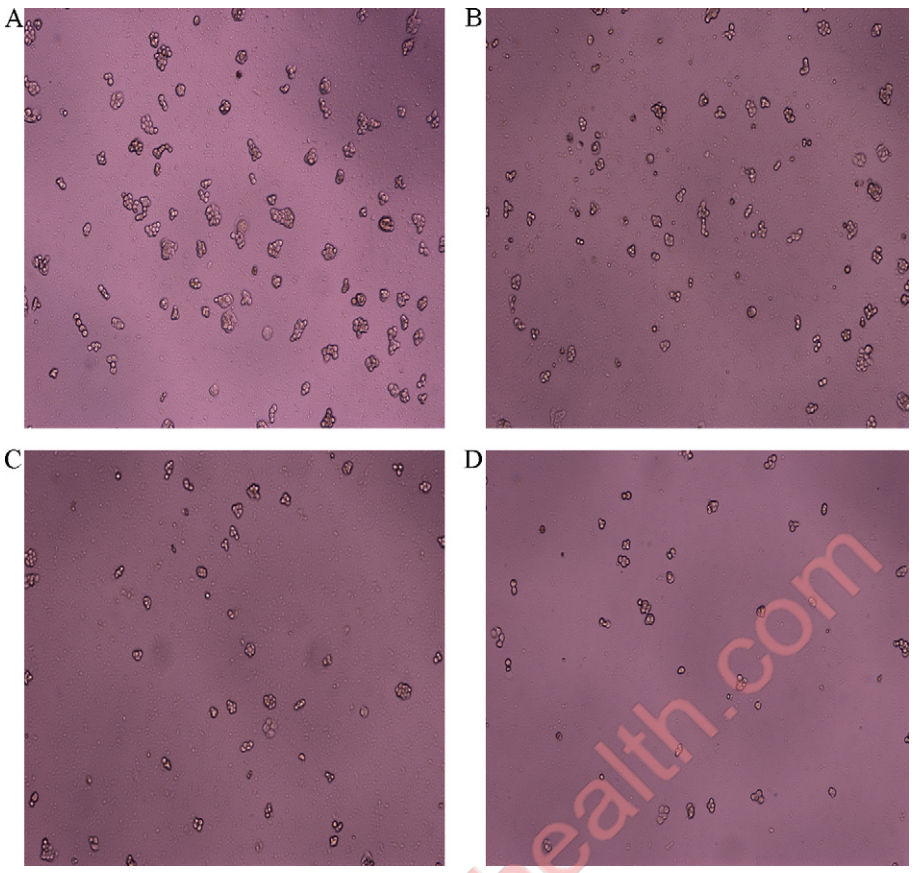

Fig. 3. The growth morphology of cells. HT-29 cells observed with a light microscope (×100) after P1 and CDDP treatment for 48 h. (A) DMSO; (B) P1 (15.625 μg/ml); (C) P1 (31.25 μg/ml); (D) CDDP (0.625 μg/ml).

As shown in Fig. 4A and B, the expression of Reg IV and EGFR were remarkably decreased, however, pIgR was evidently up-regulated with P1-treated cells at dose of 32.5 μg/ml. Although the down-regulated tendency of MCM2 and DDX32 were observed, there was no statistically significant change. These results suggest that P1 possessed prominent inhibitory properties against HT-29 cells through regulation of the functional gene expression of Reg IV, EGFR and pIgR.

3.5. Anti-tumor activity and plasma levels of PGE2, pIgR, IgA, Reg IV, EGFR and Akt

The anti-tumor effect of P1 was tested on HT-29-bearing mouse model at the dose of 50 and 100 mg/kg in vivo. As shown in Table 2 and Fig. 5, a significant tumor inhibition was observed at dose 100 mg/kg of P1 compared these in the tumor-bearing (TB) control mice. Anti-tumor activity of P1 obtained result indicated gradual increase and generated the highest inhibition ratio at 51.4% after 42 days of treatment. There was no significant difference in body weight of mice between TB control and P1treatment groups, suggesting that P1 has no toxicity to mice at the dose of 100 mg/kg (Fig. 5B). The standard reference drug (CDDP, 2 mg/kg) also exhibited a high inhibitory rate (63.42%), however, considerably decreased the body weight in HT-29-bearing mice ($P < 0.01$), and the mental conditions and coat color indicated that CDDP was harmful to TB control mice. It was noteworthy that the weight of the spleen and thymus indices also significantly increased in P1 group mice after treatment with P1 (50 and 100 mg/kg) for 42d, as compared with the TB control mice (Table 2). Therefore, it would be interesting to analyze the substantial adaptive immuno response and immunological responses. The pIgR and IgA levels in plasma were also significantly increased, but the PGE2 level was evidently

Table 2
In vivo anti-tumor activity of P1 from *Phellinus linteus*.

Sample	Inhibition degree (%)						Relative spleen weight (mg/g)	Relative thymus weight (mg/g)
	7d	14d	21d	28d	35d	42d		
TB control							4.84 ± 0.47	2.14 ± 0.16
P1 (50 mg/kg)	7.72	7.29	7.43	18.85	21.98	26.05	6.42 ± 0.38**	2.53 ± 0.19*
P1 (100 mg/kg)	22.66	21.16	27.22	27.83	40.67	51.41	6.84 ± 0.43**	2.65 ± 0.24**
CDDP (2 mg/kg)	22.91	35.01	38.50	58.98	63.42	58.54	4.07 ± 0.50	2.07 ± 0.18

TB control: tumor-bearing (TB) control; HT-29 cells were injected subcutaneously in female BALB/-nu/nu mice on day 0. The proteoglycans P1 (50 and 100 mg/kg) were dissolved in saline and TB control received 0.2 ml saline by intragastric administration everyday, CDDP (2 mg/kg, as positive control) was injected into peritoneal cavity every other day. The inhibition degree (%) is calculated according to the following formula: (average tumor volume of NaCl group − average tumor volume of sample group/average tumor volume of NaCl group) × 100%, relative spleen (thymus) weight was measured in the ratio of the spleen (thymus) weight (mg) to body weight (g). Values are mean ± S.D. ($n = 10$ each group).

* $P < 0.05$ vs. TB control.

** $P < 0.01$ vs. TB control.

Table 3
Effect of P1 from *Phellinus linteus* on levels of PGE2, pIgR, IgA, Reg IV, EGFR and Akt in plasma.

Sample	PGE2 (pg/ml)	pIgR (μg/ml)	IgA (μg/ml)	Reg IV (ng/ml)	EGFR (pg/ml)	Akt (ng/ml)
Control	118.77 ± 12.53	2.72 ± 0.39	3.14 ± 0.37	4.49 ± 0.37	281.40 ± 90.56	5.63 ± 0.58
TB control	400.77 ± 22.20**	2.11 ± 0.31*	2.09 ± 0.21**	5.29 ± 0.36*	715.51 ± 65.19**	6.62 ± 0.48**
P1 (50 mg/kg)	379.01 ± 25.26**	2.43 ± 0.49	2.36 ± 0.29**	4.93 ± 0.57	677.23 ± 70.08**	6.08 ± 0.49
P1 (100 mg/kg)	350.62 ± 20.30**,++	2.57 ± 0.26+	2.55 ± 0.23**,++	4.75 ± 0.35+	619.92 ± 40.64**,+	5.69 ± 0.80+
CDDP (2 mg/kg)	362.87 ± 17.39**,++	2.27 ± 0.43	2.21 ± 0.19**	4.96 ± 0.37	686.1 ± 95.25**	6.15 ± 0.41

TB and positive control and the proteoglycan (P1)-groups were inoculated HT-29. The mice were supplied orally daily with NaCl, P1 (50 and 100 mg/kg) or injected CDDP (2 mg/kg) into peritoneal cavity every other day. Plasma was collected from the tumor-bearing mice after 42 days. The biochemical parameters were determined using ELISA. Results are presented as mean ± S.D. (n = 10 each group).

* $P < 0.05$ vs. normal.
** $P < 0.01$ vs. normal.
\+ $P < 0.05$ vs. TB groups.
++ $P < 0.01$ vs. TB groups.

decreased in colorectal carcinoma model mice compared with the HT-bearing model (Table 3). The thymus is a specialized organ in which the T lymphocytes develop, differentiate, and mature, while spleen contains T-cells and B-cells [15,16]. The present results indicated that P1 is a potent immunomodulating and immunoenhancing agent, which is in accordance with previous reports of some of the functions of various polysaccharides [15,16]. Moreover, statistically significant decreases of the Reg IV, EGFR and Akt concentrations in plasma measured by ELLISA were observed (Table 3). These results were consistent with our foregoing RT-PCR results measured in HT-29 cells in vitro, suggesting that P1 also could directly inhibit colorectal carcinoma via disrupting the Reg IV/EGFR/Akt signaling pathway.

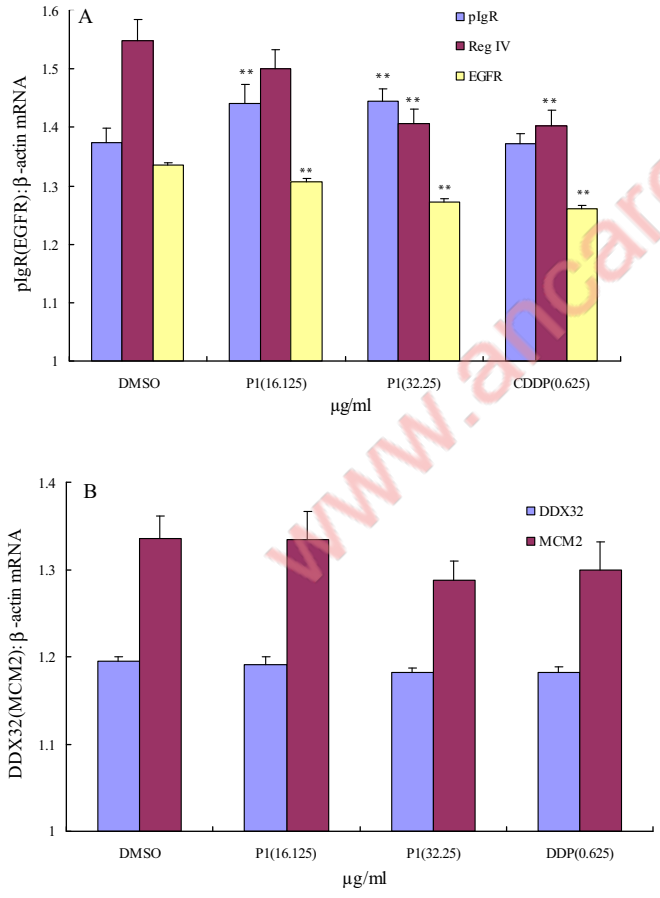

Fig. 4. Analysis of relative changes of pIgR, Reg IV, EGFR(A), DDX32 and MCM2(B) mRNA levels in HT-29 cells following treatment with P1 (15.625 and 31.5 μg/ml) and CDDP (0.625 μg/ml) for 48 h as detected by real-time RT-PCR. All expression data were corrected by the control gene β-actin. Standard error bars were calculated based on the mean of three replicate treatments. Statistically significant differences compared to the DMSO control were determined by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) and are indicated by **$P < 0.01$.

4. Discussion

This study demonstrated that the proteoglycan (P1) from *P. linteus* (PL) has an antiproliferative effect on HepG2, HT-29, NCI-H 460 and MCF-7 human colon cancer cells, especially good effect on HT-29 cells. Growth inhibition of HT-29 cells by P1 treatment is mediated through the induction of immunoreaction and apoptosis. Further studies found that the immune function of P1 may be associated with increasing spleen and thymus relative weights, enhancing the mucosal IgA response by up-regulating the expression of pIgR and allowing T cells escape PGE2 attack through decreasing plasmatic PGE2 level. We also demonstrated for the first time that P1 could inhibit HT-29 cells proliferation, suggesting that P1 plays an important role in mediating the proliferative action stimulated by down-regulating the Reg IV and EGFR, and may lead to a disruption of the Reg IV/EGFR/Ak signaling pathway [12]. Further research is needed to better understand the Reg IV, EGFR and Akt levels in plasma in vivo. So we expect that P1 can be used as an adjuvant chemotherapeutic and chemopreventive agent as well as immunostimulant against the potential threat of colonic carcinoma.

Mushroom polysaccharides are increasingly being used to treat a wide variety of diseases. Previous reports showed that the polysaccharides and proteoglycans from PL had inhibitory effects on tumor growth and metastasis in a murine model [1], and also could inhibit the proliferation and colony formation of SW480 human colon cancer cells [3]. But whether all the polysaccharides from PL have the anti-tumor activities and which cancers are suitable to treatment draw our attention. In this study, a significant tumor inhibitory effect of P1 from PL on HepG2, HT-29, NCI-H 460 and MCF-7 human colon cancer cells was observed, but the cytotoxicity to normal cells (L-929) was also evident at high doses. Although previous researches deemed that protein-bound polysaccharides are thought to have no cytotoxicity on cancer cells, recently increasing studies reported that the proteoglycan could kill cancer cells directly when the doses were high [3]. Our results showed that the P1 has an effective inhibition on HT-29 cells under 62.5 μg/ml, which was a dose of non-toxic to normal cells.

To data, there are a large number of studies on anticancer activities of polysaccharides and their molecular mechanisms have been documented. Previous studies investigated the anticancer mechanisms of polysaccharides have focused upon the large part on the functional activations of immune cells, such as T cells, B cells, nat-

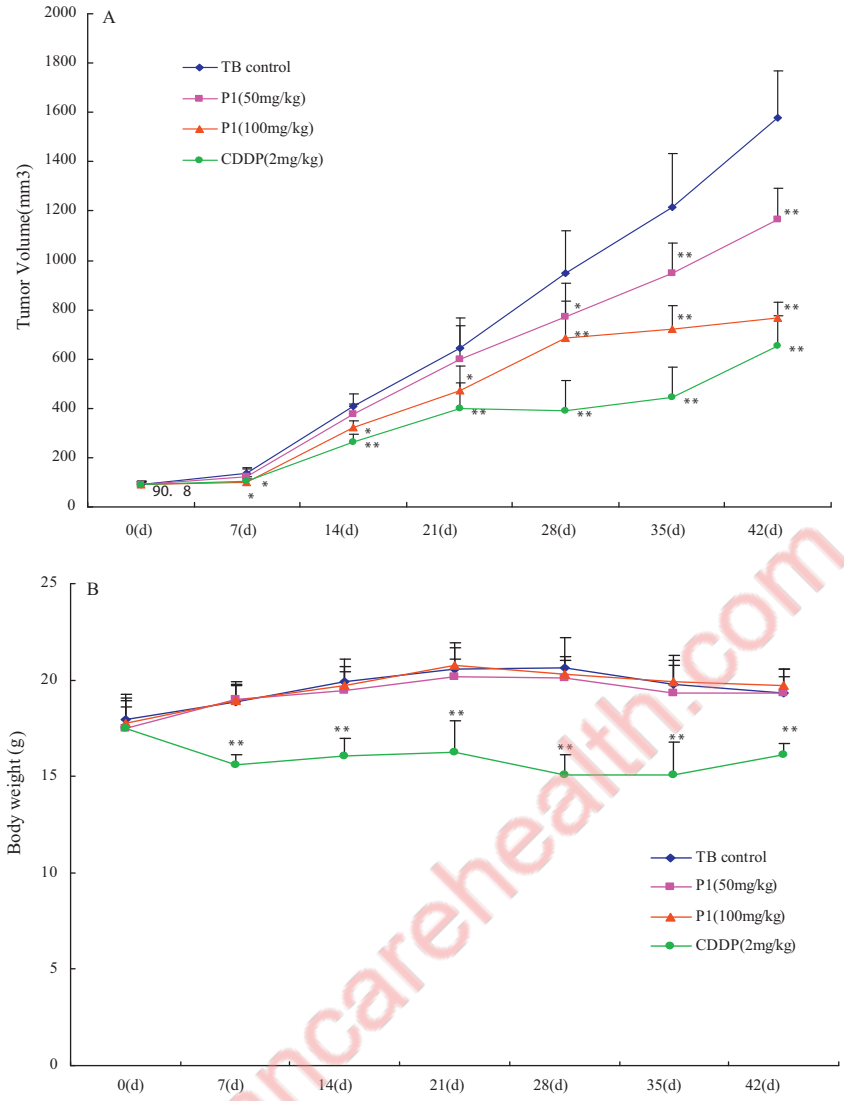

Fig. 5. The anti-tumor activity of P1 from *Phellinus linteus* in vivo. TB control: tumor-bearing (TB) control. HT-29 cells were injected subcutaneously in female BALB/c-nu/nu mice on day 0. The proteoglycans P1 (50 and 100 mg/kg) were dissolved in saline and TB control received 0.2 ml saline by intragastric administration everyday, CDDP (2 mg/kg, as positive control) was injected into peritoneal cavity every other day. The maximal (a) and minimum (b) diameters of solid tumors (A) and the body weights (B) of mice were measured every week. The tumors volume was calculated as $V = a \times b^2/2$. Compared with the TB group, Values are mean ± S.D. ($n = 10$ each group). $^*P < 0.05$ and $^{**}P < 0.01$ vs. TB control.

ural killer cells, dendritic cells, and macrophages, which all have the capability to eliminate transformed cancer cells [2,17–19]. In our study, we found that P1 has a direct antitumor effect through inducing apoptosis and inhibiting the karyokinesis of HT-29 cells, the results are consistent with the previous reports by Li et al. [3]. Meanwhile, the highest inhibition ratio of 51.4% after 42 days treatment with P1 also observed in HT-29-bearing model mice. In an attempt to clarify the potential mechanism(s), the effects of P1 on the relative spleen and thymus weights were measured, and the expression patterns (up/down-regulation) of many functional genes (MCM2, DDX32, pIgR, Reg IV and EGFR) in the plasma of colorectal carcinoma were also detected. The relative thymus and spleen weight were important indices for nonspecific immunity. The increase in thymus and spleen indices of mice treated with P1 indicated that P1 is a potent immunomodulating and immunoenhancing agent. It is well known that the overproduction of PGE2 has been associated with numerous pathological conditions including chronic inflammation and colon carcinogenesis. PGE2 from colon cancer cells can inhibit T cell response [20], impair differentiation and function of dendritic cells by suppressing poly (I:C)/LPS mediated IFN-α and IL-12 production [21]. In addition, PGE2 from colon cancer cells can also induce generation of regulatory T cells [20] and myeloid-derived suppressor cells, which allow the tumor cell to escape of immune surveillance [22]. IgA refer to as the first line immune defense, protecting mucosal surfaces against environmental and sexually transmitted pathogens [23]. Several investigators have observed an increased mucosal IgA response by long-term treatment with multiple polysaccharides [24,25]. The pIgR is a transmembrane glycoprotein expressed on secretory epithelial cells, which mediates the transport of polymeric immunoglobulins, primarily polymeric IgA (pIgA), into external secretions. Up-regulation of pIgR expression increases the capacity of mucosal epithelial cells to transport pIgA, thus enhancing the functions of IgA [26]. So the expression of pIgR is regulated with respect to the effects of specific immune modulators [17]. Our findings show that P1 could up-regulate the pIgR mRNA level in HT-29 cells (Fig. 4A). Correspondingly, the pIgR level change in colorectal carcinoma model mice was in well accordance with the RT-PCR results in vitro (Table 3), especially, the increased IgA concentration suggested that P1 exert their effects, at least part through protect-

ing T cells from escaping PGE2 attack and enhancing the mucosal IgA response.

Reg IV, a dominant member of the Reg multigene family, is of considerable interest because of its up-regulated expression in the colorectal carcinoma (CRC) and colon adenocarcinoma cell lines [27,28]. Recently, Bishnupuri et al. reported that Reg IV protein is a potent activator of the EGFR/phosphoinositide 3-kinase/Akt/AP-1 signaling pathway in human colon cancer cell lines [12]. Activation of EGFR signaling pathway results in mitogenic signaling in colon cancer cells, increase cell proliferation, angiogenesis, metastasis and decreased apoptosis [13,29,30]. Interestingly, Nanakin et al. found that EGF and TGF-α enhanced Reg IV gene expression by ERK signaling pathway in SW403 cells line [31]. Taken together, these studies suggest that a positive expression feedback loop between EGF and Reg IV exists in the signaling pathways. So disruption of Reg IV/EGFR signaling may be used as a therapeutic intervention for human gastrointestinal adenocarcinomas. Many researchers reported that fucoidans from *Laminaria gurjanovae* mainly consisting of glucose, fucose, galactose and mannose could bind to vascular endothelial growth factor 165 (VEFF165) and its receptors [32,33]. Recently, Lee et al. (2008) found that fucoidan could bind with EGF, blocked EGF-induced phosphorylation of EGFR, resulting to inhibit EGF-induced cell transformation [34]. Our studies showed that treatment of colonic adenocarcinoma cells with P1 resulted in a significant dose-dependent inhibition in cell numbers (Fig. 2) and cells' mitosis (Fig. 3), so we identified the expression of Reg IV and EGFR in P1-treatment HT-29 cells. RT-PCR results showed that P1 had the capacity to down regulate the expression of Reg IV and EGFR (Fig. 4A), these effects may be important for its growth inhibitory capacity to HT-29 cells. Therefore, we hypothesized that the proteoglycan P1 composed of L-fuc, D-gal, D-glu, D-man and other monosaccharides may bind with EGF as fucoidan, blocked EGF-induced phosphorylation of EGFR and feedback loop induced Reg IV down-regulation, thereby inhibiting intracellular signaling and decreasing the expression of EGFR without activation. In order to testify this hypothesis, HT-29 cells were inoculated in mice to establish the colorectal carcinoma model. The differences in the P1 dose intake for 42 days significantly lower the tumors volume, indicating that P1 has an anti-tumor effect in vivo (Table 2 and Fig. 5), decrease of Reg IV, EGFR and Akt levels in plasma (Table 3) which strongly supports that the P1 inhibited colorectal carcinoma by disrupting the Reg IV/EGFR/Akt signaling pathway. Although MCM2 and DDX32 play pivotal roles in tumor growth, unfortunately, no significant change in their levels were noted in HT-29 cells after P1-treatement (Fig. 5).

In summary, the polysaccharide (P1) from *P. linteus* is an immunostimulator that has therapeutic activity against cancers. Here, we show that P1 markedly inhibits the growth of HT-29 cells and directly effect on cancer cells' mitosis and proliferation through down-regulate the Reg IV and EGFR, and upregulating the expression of pIgR. In HT-29-bearing model mice, the significant changes of plasma biochemical parameters approved P1 inhibitory colorectal carcinoma effect. These results suggest that P1 has two antimetastatic functions, it could act not only as an immunopotentiator, but also as an anti-Reg IV/EGFR/Akt signaling pathway agent inhibiting HT-29 tumor directly.

Acknowledgements

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Effects of Fermented Rice Wine by Using Mycelium of *Phellinus linteus* on the Expression of Inflammation-Related Proteins in Human Hepatoma Cells and Rat Liver

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We have recently discovered that mycelium of *Phellinus linteus*, popular medical mushrooms in Korea, possess alcohol dehydrogenase and produce alcohol. In the present study, it was examined that the effect of fermented rice wine made by using mycelium of *P. linteus* (FLMP) on the expression of inflammation-related proteins in both $HepG_2$ cells and rats. To examine the effect of FLMP on the morphology and expression of inflammatory proteins in $HepG_2$ cells, the cells were incubated with ethanol, and FLMP for 24 hours, and then analyzed by microscopic observation and Western blot and reverse transcription polymerase chain reaction (RT-PCR). While ethanol induced the morphological change accompanied with cell debris formation and scattering on $HepG_2$ cells, FLMP had no effect. The results of Western blot and RT-PCR analyses showed that the level of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-1 and COX-2 was induced by ethanol, however, FLMP inhibited the expression of these proteins and its mRNAs. In the animal model, the value of glutamate oxaloacetate transaminase and glutamate pyruvate transaminase was significantly increased by administration with ethanol. But the group administrated with FLMP showed lower levels on the changes of these markers compared with ethanol-administrated group. Besides, the results of Western blot and RT-PCR analyses showed that the expression of inflammatory proteins such as iNOS, COX-1 and COX-2 was not affected by FLMP administration in rat liver. About histopathological and immunohistochemical observations, inflammatory loci were markedly decreased in the FLMP-administrated rat compared to ethanol-administrated rats and showed weaker COX-2 and iNOS immunoreactions. These results suggested that FLMP showed slight changes on the inflammatory proteins expression compared to ethanol and FLMP may be used as a functional alcoholic beverage.

Key words – *Phellinus linteus*, wine, inflammation, liver

The *Phellinus linteus*, commonly referred to as Sangwhang in Korea, is well known as one of the most popular medical mushrooms due to its high anti-tumor and immunostimulating activities[6,11]. Aqueous extract from the fruiting body or mycelia of *P. linteus* has been reported to produce anti-tumor, anti-angiogenic, anti-mutagenic and immunomodulatory activities *in vivo* and *vitro*[3].

Despite such great medical value, *P. linteus* is restricted to use by reason of it being extremely rare to find in the nature and it's high price. During mass-culture of mycelia of this fungus, we have discovered that mycelium of *P.linteus* possesses some alcohol dehydrogenases and produces alcohol. Most alcoholic beverages are made by the fermentation process involving the action of various yeasts [13]. The fermented rice wine is made by using mycelium of *P. linteus* (FLMP) is the first alcoholic beverage without alcoholic fermentation by yeast or any kind of microorganism. Furthermore, if the FLMP is beneficial as *P. linteus*, this rice wine may be a new type of healthy functional rice wine.

However, all kinds of alcoholic beverages have an injurious effect on the function of the liver and other organs. Thus it is necessary to confirm the effect of FLMP on the inflammatory proteins and liver function markers. The present study was designed to examine the effects of FLMP on the expression of inflammation-related proteins and the change of functional markers in human hepatocarcinoma cells and rat livers.

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Materials and Methods

Production of FLMP

To increase ethyl alcohol production the rice-based medium was used, and *P. linteus* was co-cultured with *Aspergillus oryzae*. First, *A. oryzae* was aseptically inoculated on 1.2 kg of autoclaved rice. When the spawned rice had been fully colonized with *A. oryzae*, 3 kg of autoclaved rice and 90 g (wet weight) of *P. linteus* mycelia were mixed all together in 5 liters of autoclaved water. Incubation was done for 11 days at 25℃ without shaking. Each 1.5 kg of autoclaved rice was additionally added at 24 h and at 72 h of the incubation period. The FLMP has 14% alcoholic concentration.

Antibodies and chemicals

Antibodies against cyclooxygenase (COX)-1, COX-2 and inducible nitric oxide synthase (iNOS) and secondary horseradish peroxidase-conjugated rabbit immunoglobulin G antibodies were obtained from Santa Cruz Biotechnology (CA, USA). All other chemicals were obtained from Sigma (MO, USA).

Cell culture and treatment

$HepG_2$ cells were obtained from Korean Cell Line Bank (Seoul, Korea) and were grown in DMEM (Dulbecco's Modified Eagle Medium, GIBCO BRL, NY, USA) supplemented with 10% fetal bovine serum (GIBCO BRL) and 1% penicillin-streptomycin (GIBCO BRL). $HepG_2$ cells have been widely employed to analyze in vitro alcoholic disease model systems. The cells were cultured at 37℃ in a 5% CO_2 atmosphere. Cells were seeded and stabilized for 24 h and then, treated with a fresh medium containing 14% ethanol or FLMP for 24 h.

Ethanol and FLMP administration

Male Sprague-Dawley rats, weighing about 120 g on average, were obtained from Hyochang Science Co. in Korea. Rats were housed under conditions of 22℃ and 12 h dark and light cycle, were fed a commercial diet, and allowed tap water *ad libitum* starting 2 weeks before and throughout the study. Rats were administrated orally 5 ml of 14 % ethanol, FLMP and PBS twice a day for 10 days.

SDS-PAGE and western blot analysis

Cells were washed in a cold phosphate-buffered saline and lysed in lysis buffer (40 mM Tris-Cl pH 8.0, 120 mM NaCl, 0.1% NP-40, 0.1 mM sodium orthovanadate, 2 μg/ml leupeptin and 100 μg/ml phenyl methylsulfonyl fluoride) at 4℃ for 30 min. The lysates were centrifuged at 14,000 rpm for 30 min at 4℃, and the supernatants were served as whole cell protein extracts. Rat livers were washed in cold HEPES buffer, and homogenized in 9 volumes of potassium HEPES buffer containing 0.5% Triton X-100, 1 mM DTT, 5 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 mM phenyl methylsulfonly fluoride. The homogenates were centrifuged at 14,000 rpm for 30 min at 4℃ and the supernatants served as liver protein extracts. Equal amounts of proteins were separated by 8-12% SDS-PAGE. The resulting gels were transferred to immobilon-P transfer membranes (Millipore, Bedford, MA, USA). For Western blotting, the membranes were incubated with the specific first antibodies for 2 h at room temperature, and then the blots were incubated with horseradish peroxidase-conjugated secondary antibody. The antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham, Arlington Heights, IL, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

For mRNA analysis, cells and rat livers were washed in cold phosphate-buffered saline and homogenized in TRIzol reagent (Invitrogen) following the manufacturer's manuals. mRNA levels were determined by RT-PCR using the GeneAmp RNA-PCR kit (Perkin Elmer) with 50-100 ng of poly A^+ RNA and specific primers as previously described [1]. 10 μl of the reaction products were subjected to electrophoresis in 1% agarose gel and visualized by ethidium bromide (EtBr) staining.

Serum analysis

For the serum analysis, the blood was collected from the heart and immediately centrifuged at 3,000 rpm for 25 min and the plasma was stored at -20℃ for later analysis. Levels of serum glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were determined by the Cobas Mira (Roche, Switzerland).

Histopathology and immunohistochemistry

The livers were fixed in 4% paraformaldehyde in PBS for 18 h and dehydrated in a graded ethanol series. After embedment in paraffin, serial 5 μm thick sections were prepared. For histopathological examinations, hematox-

ylin-eosin stain and periodic acid Schiff's (PAS) reaction were used. After deparaffinized in 58℃ xylene, the sections were exposed to 0.3% methanolic hydrogen peroxide for 30 min, and washed with PBS. Tissues were then treated with goat normal serum at room temperature for 30 min followed by treatment with anti-iNOS, COX-1 and COX-2 diluted for 1:500 in moisture chamber at 4℃ for 16 h. After being washed by PBS, tissues were incubated with the secondary antisera, biotinylated anti-rabbit IgG for 30 min and washed with PBS. These sections were further incubated in avidin-biotin-peroxidase complex kit (Vector Lab. CA, USA) at room temperature for 1 h. Diaminobenzidine substrate kit for peroxidase (Vector Lab. CA, USA) was applied. For the controls, treatment with primary and secondary antibodies was omitted.

Results

Effect of FLMP on the morphology of $HepG_2$ cells

It was examined the effects of FLMP compared with ethanol on the morphology of liver cell. $HepG_2$ cells were treated with ethanol or FLMP including 0.28%, 0.56% and 1.4% of alcoholic concentration for 24 h. As shown in Fig. 1, ethanol induced morphological change including scattering and cell debris formation, while the cells treated with FLMP had no change similar to the control. The result suggests that FLMP have some components which are able to protect cellular morphology or structure from its own alcohol.

Effect of FLMP on the expression of inflammation-related proteins in $HepG_2$ cells

To investigate the effect of FLMP on the expression of inflammation-related proteins in $HepG_2$ cells, the cells were treated with 0.28% of alcoholic concentration of ethanol and FLMP for 24 h. As shown in Fig. 2A, expression of COX-1 and COX-2 was induced in $HepG_2$ cells treated ethanol than FLMP. Also, iNOS expressions were strongly induced in $HepG_2$ cells treated ethanol than FLMP. This alleviative effect of FLMP on the protein expression was confirmed by RT-PCR analysis (Fig. 2B). The results indicated that FLMP lowered the induction of inflammation more than ethanol through decreasing the inflammatory

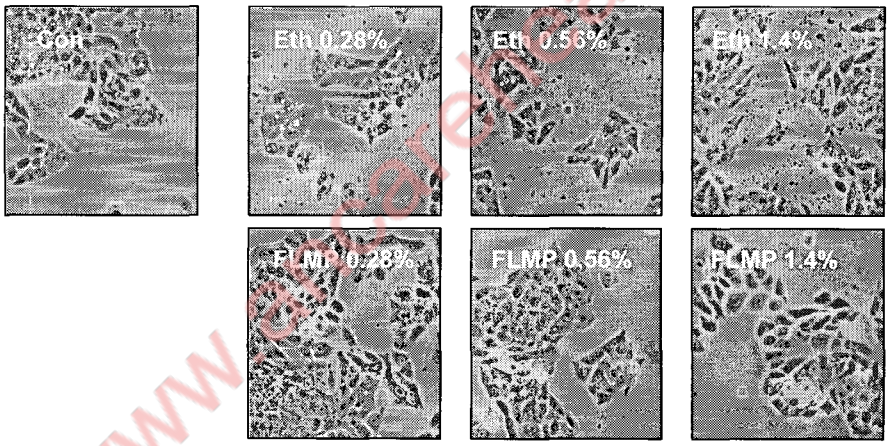

Fig. 1. Morphological changes in $HepG_2$ cells following incubation with ethanol and FLMP. Cells were treated with ethanol and FLMP of various alcoholic concentrations for 24 h (×200).

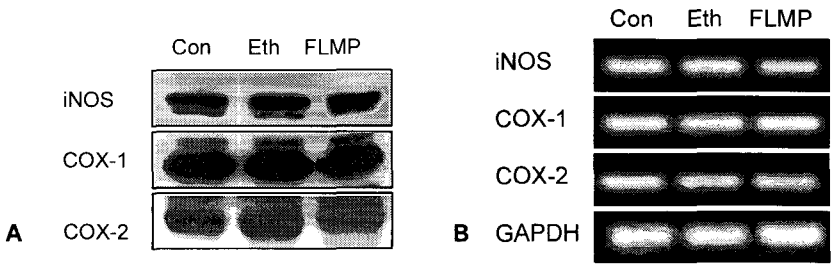

Fig. 2. Effects of ethanol and FLMP on the expression of inflammation-related proteins (A) and mRNAs (B). $HepG_2$ cells were incubated with ethanol and FLMP (0.28% alcohol each) for 24 h. The expression levels of iNOS, COX-1 and COX-2 protein and mRNA were examined by Western blot and RT-PCR as detailed in Materials and Methods.

protein expression.

Effect of FLMP on the liver function in rats

In the results of *in vitro* experiment, ethanol could induce morphological change and inflammation in $HepG_2$ cells, however, FLMP showed little marked effects. Therefore, the effect of FLMP on the liver function was examined in the animal model. Rats were administrated orally 5 ml of ethanol, FLMP or PBS twice a day for 10 days. After treatment, the markers for liver function including serum GOT and GPT were examined. As shown in Fig. 3, the level of GOT was increased in the ethanol-administrated group compared with control, but there was no significant changes. However, GPT level was significantly increased in ethanol-administrated group compared with control and this level also markedly decreased in the FLMP-administrated group compared with the ethanol-treated group.

Effect of FLMP on the expression of inflammation-related proteins in the rat liver

Fig. 3 suggests that ethanol has injurious effects on the liver function markers relating to inflammation in the liver. Then inflammation-related proteins in the rat liver administrated with ethanol were examined. As shown in Fig. 4A, iNOS, COX-1 and COX-2 protein was increased in the rats administrated with ethanol, but these proteins, especially iNOS, were reduced in the rats administrated with FLMP. To confirm these results, RT-PCR analysis was performed as shown in Fig. 4B.

Histopathological and immunohistochemical observation of rat liver

Inflammatory loci with stromatic changes including

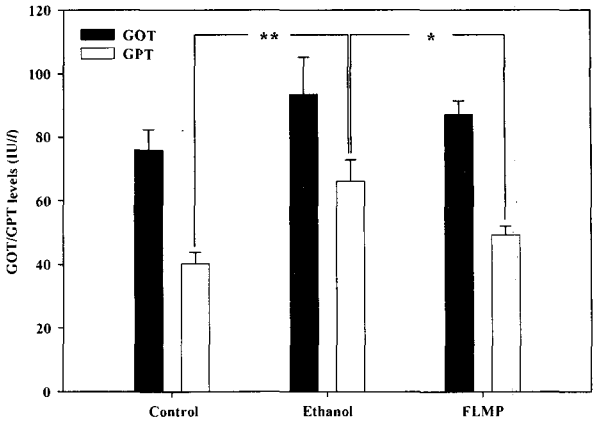

Fig. 3. Effects of ethanol and FLMP on the serum GOT and GPT levels. Rats were administrated with 14% ethanol and FLMP (14% alcohol content) twice a day for 10 days and examined by a blood serum analysis instrument. Values are means±SEM of eight animals. *, $P<0.05$; **, $P<0.005$.

cloudy swelling, hydropic degeneration and inflammatory cells infiltration were markedly increased in the ethanol-administrated rat. But a similar histopathological pattern with PAS reaction showed in rats administrated with FLMP compared with control ones (Fig. 5). The iNOS and COX-2 immunoreaction of hepatocytes, especially in the inflammatory loci, were increased in ethanol-administrated groups compared to control ones. But these immunoreactions were decreased in the rats administrated by FLMP with fewer inflammatory loci (Fig. 6).

Discussion

Generally, *P. linteus* has been used as a medicinal mushroom for the treatment of inflammatory disease and cancer in Korea. The investigations for the crude extract and chemical constituents of this fungus were also focused on

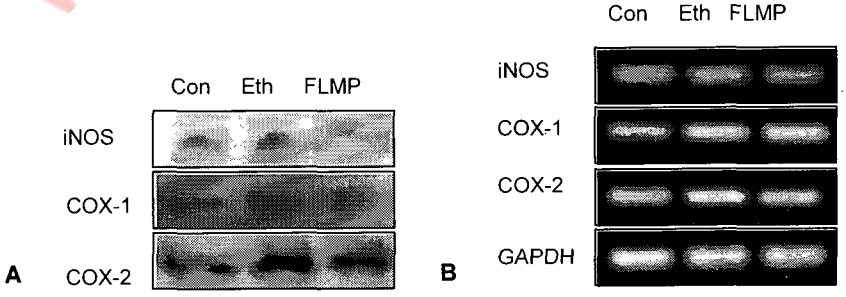

Fig. 4. Effects of ethanol and FLMP on the expression of inflammation-related proteins (A) and mRNA (B) in rat liver. Rats were administrated with 14% ethanol and FLMP (14% alcohol content) twice a day for 10 days and examined by Western blot and RT-PCR analysis in rat liver.

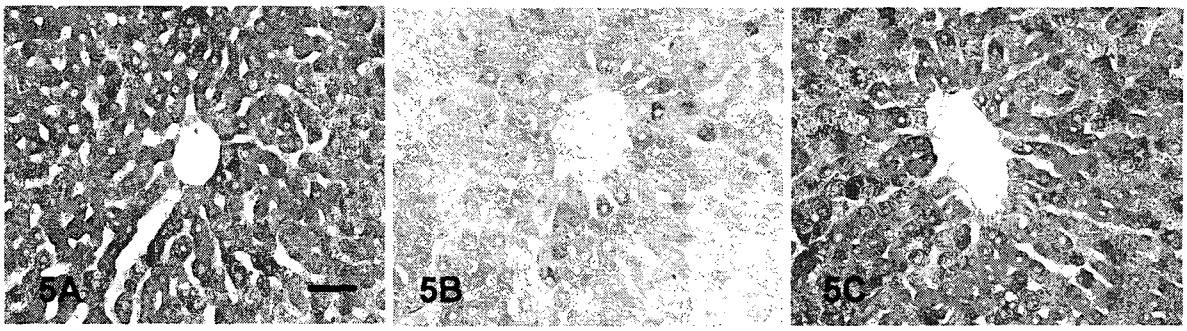

Fig. 5. Photomicrographs showing the liver of the rat in the control (A), ethanol- (B) and FLMP-administrated rats (C). Note severe inflammatory loci and a marked weak PAS reaction of the ethanol-administrated rats compared with the control and FLMP-administrated ones. Scale bar=50 μm.

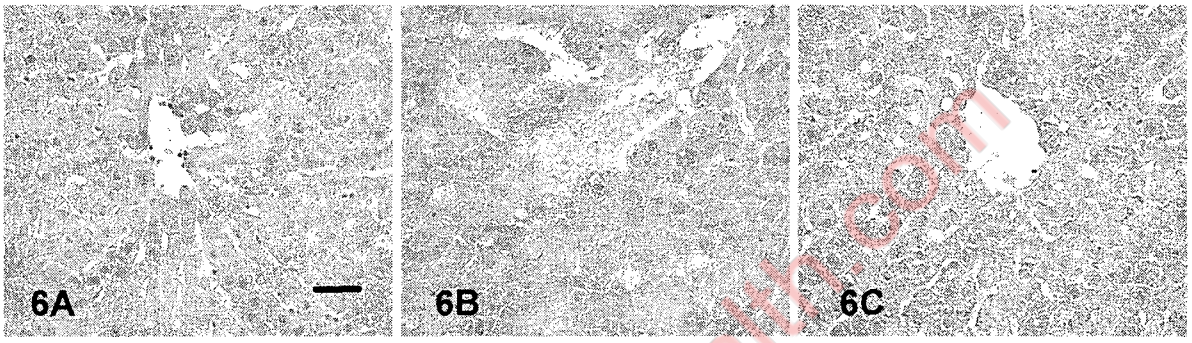

Fig. 6. Immunoreaction of COX-2 in the liver of control (A), ethanol- (B) and FLMP-administrated rats (C). Note decline of COX-2 immunoreaction, especially in inflammatory loci, in the FLMP administrated rats compared with ethanol-administrated ones. Scale bar=50 μm.

their anti-tumor activities. Like fruiting bodies of *P. linteus*, its mycelial extract was found to contain potent anti-tumor activities[3] and showed anti-tumor activity toward solid tumors planted in mice[11]. The polysaccharides of mycelial culture of *P. linteus* stimulate humoral and cell-mediated immunity and exhibit a wider range of immunostimulation and anti-tumor activity[5].

The liver is one of the major organs to be damaged during the heavy intake of alcohol. Many studies have demonstrated that liver disease resulted from the dose-and time-dependent consumption of alcohol[4,8]. We have firstly observed that the effects of FLMP on the morphological change of $HepG_2$ cells in the present study. $HepG_2$ cells treated with FLMP showed a similar morphology as like the normal cells. These results suggest that FLMP may have some components to protect cellular morphology or structure from its own alcohol.

The liver injury in response to alcohol is associated with an inflammatory response as an important precursor to the development of irreversible liver disease[10]. The iNOS is a member of the nitric oxide synthases family and hepatic injury is associated with up-regulation of this enzyme expression[9,14]. COX-1 is constitutively expressed in most cells and tissues, whereas COX-2 is only expressed when induced by inflammatory stimuli[16,17]. COX-2 is selectively expressed in response to various inflammatory stimuli and is increased in adjacent cirrhotic tissue of hepatocellular carcinoma[2,12].

In the present study, higher expression of iNOS, COX-1 and COX-2 were induced in the ethanol treated $HepG_2$ cells compared with FLMP-treated cells (Fig. 2). Furthermore, *in vivo* experimental data confirmed that FLMP could affect an expression of inflammatory proteins in rat liver (Fig. 4). The immunohistochemical analysis also showed a similar result with mRNA and protein expression. The declines of iNOS and COX-2 immunoreaction with a decrease of inflammatory loci in an FLMP-administrated rat were observed compared to ethanol-administrated ones (Fig. 6).

The GOT and GPT levels are correlated with the degree of inflammation or cell death of the liver[7,15]. Therefore, it was investigated that the effects of FLMP on the alteration of GOT and GPT levels in rat blood serum served as

a liver functional marker. Lower levels of these enzymes, especially GPT, were demonstrated in the serum of FLMP-administrated rats compared to ethanol-administrated ones with histological changes such as fewer inflammatory loci (Figs. 3 and 5).

Previous studies showed that the methanolic extract of the mycelial culture of *P. linteus* significantly protects against hepatotoxins-induced toxicity in primary cultured rat hepatocytes[6]. It suggested that alcohol causes liver damage through hapatocytic necrosis including inflammatory process, but some components of FLMP may prevent alcohol-induced liver injury.

Although exact components of this beverages are not clear, active fraction of FLMP was composed of 68±9% of protein and 15±4% of sugar. It's constitute of sugars were Glu:Gal:Man:Fru=46.6:12.8:26.4:3.8 in molar ratio. The ^{1}H, ^{13}C-NMR analysis showed that the main glucan part of the extract of FLMP was a mixtures α-D-glucan and β-D-glucan (unpublished data). Though FLMP has relative low alcohol content (14%), FLMP can also cause the alcoholic liver disease. However, FLMP administration induced lower expression of inflammation-related proteins in both $HepG_2$ cell and rat liver compared with ethanol. Furthermore, FLMP-administrated groups showed slight changes on the liver enzyme abnormalities. This was also confirmed by histopathological and immunohistochemical observations in rat liver.

As shown in a previous report that anti-tumor polysaccharides from mushrooms are β-D-glucan[11], the polysaccharides of FLMP may prevent alcohol-induced liver injuries. Consequently, these results suggest that FLMP may have some components to hepatoprotective activities. Then these data will be a useful additional source of information about mycelium of *P. linteus* and a good guide for the developments of the other functional alcoholic beverages.

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초록 : 상황버섯 균사체를 이용한 발효주가 인체간암세포와 흰쥐 간의 염증관련 단백질 발현에 미치는 영향

안성민 · 이준혁 · 최영현[1] · 이용태[2] · 정경태[3] · 정영기[3] · 조운복[4] · 최병태*
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상황버섯균사체를 이용한 발효주 (FMLP)가 인체간암세포와 흰쥐 간에서 염증관련단백질의 발현과 간 손상 관련효소에 미치는 영향에 대해 조사하였다. 에탄올, FLMP를 인체 간암세포인 $HepG_2$세포에 24시간 처리한 경우, 대조군에 비해 에탄올을 처리한 세포는 농도가 증가할수록 세포의 형태 변화가 심한 반면 FLMP를 처리한 세포는 알코올의 농도가 증가해도 세포의 형태 변화를 관찰할 수 없었다. $HepG_2$세포의 염증 관련 단백질 발현을 보기 위해 Western blot과 RT-PCR을 한 결과 COX-1, COX-2, iNOS, TNF-α의 발현이 에탄올에 의해 현저하게 유도되었으나, FLMP는 $HepG_2$세포에서 mRNA와 단백질의 발현이 에탄올에 비해 억제되었다. 흰쥐를 이용한 동물실험에서 간 기능의 지표가 되는 혈청내 GOT와 GPT양 등이 에탄올 투여 그룹에서 현저히 증가했으나 FLMP를 투여한 그룹에서 에탄올 투여 그룹에 비해 낮게 나타났다. 면역조직화학적 분석에서 에탄올을 투여한 쥐의 간세포에서 iNOS와 COX-2의 발현이 현저히 증가하나 FLMP를 투여한 쥐의 간세포에서 미약한 면역반응이 관찰되었다. 이상의 결과로 보아 FLMP는 동일한 농도의 에탄올에 비하여 간 손상의 가능성이 적은 것으로 사료되며 이 자료들은 상황버섯에 대한 연구와 다른 기능성 주류에 대한 기초 자료가 될 것이다.