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Full Length Research Paper

The antitumor activities of Lentinula edodes C_{91-3} mycelia fermentation protein on S_{180} (Mouse sarcoma cell) *in vivo* and *in vitro*

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Lentinula edodes is one of the most widely used medicinal mushrooms which exhibit significant antitumor activity. It is mostly studied and several studies have showed that its polysaccharides play critical roles in the antitumor effects. In this study, we determine the antitumor activity of Lentinula edodes C_{91-3} mycelia fermentation protein (LFP_{91-3}) on S_{180} tumor cells *in vivo* as well as *in vitro*. The results showed that LFP_{91-3} inhibited the growth of S_{180} tumor cells *in vitro*, and the mechanism may be related to apoptosis induced by LFP_{91-3}. *In vivo*, LFP_{91-3} treatment significantly prolonged life span in S_{180} tumor-bearing mice. LFP_{91-3} can be used as an important antitumor element in Lentinula edodes C_{91-3} mycelia fermentative liquid.

Key words: Lentinula edodes C_{91-3} fermentation protein, Lentinula edodes, antitumor activity.

INTRODUCTION

Lentinula edodes, commonly known as the Shiitake mushroom, is the second most popular and widely cultivated edible mushroom in the world. As the main medicinal fungi, its extract has anti-viral, anti-bacteria, anti-tumor activity and regulation of immune function, etc (Hearst et al., 2009; Kuppusamy et al., 2009; Sarangi et al., 2006; Unursaikhan et al., 2006). The strain of Lentinula edodes was studied in March, 1991. So we named Lentinula edodes C_{91-3}, and "C" means China. Numerous studies showed Lentinan could inhibit tumor cells growth, improve patient's symptoms, and reduce adverse reactions. It is now officially used for clinical medicine as adjuvant chemotherapy drugs (Ooi and Liu, 2000; Kidd, 2005; Zheng et al., 2005; Fang et al., 2006; Miyaji et al., 2006). However, antitumor activity of Lentinula edodes on protein level was rarely reported. In this study submerged fermentation for Lentinula edodes C_{91-3} mycelia was done and its antitumor effects on protein level were determined.

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MATERIALS AND METHODS

Experimental animals

50 healthy inbred BALB/C mice were selected. Three months old SPF-grade mice were used having weight 18 to 20 g. We used 20 male and 20 female mice, obtained from the Experimental Animal Center of Dalian Medical University. This study was approved by Dalian Medical University Animal Ethical Review Committee, similar to the guidelines for the use and care of animals that are published by the National Institute of Health.

Carcinoma cell lines

S_{180} (mouse sarcoma tumor) cell lines were obtained from the Department of Microbiology of Dalian Medical University. Cell lines were grown in RPMI-1640 medium (Gibco) containing 10% fetal calf serum (China National Medicines Corporation, Ltd). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Lentinula edodes C_{91-3} strain and culture conditions

Lentinula edodes C_{91-3} strain were obtained from the Department of Microbiology of Dalian Medical University. It was cultured in the potato culture medium containing 1% Vitamin B1, 2.0% Agar, 0.15% $MgSO_4$, 0.3% K_2HPO_4, and 2.0% glucose having pH 6.0.

Table 1. Comparison of survival times of S_{180} tumor-bearing mice among the 5 treatment groups.

Group	Death/total	Mediated life span(d)	Survival rate (%)
NS	10/10	15.80±2.94	0
CTX	10/10	21.30±2.50*	0
LFP91-3I	8/10	23.00±2.62*	20
LFP91-3II	6/10	28.50±4.60♦*	40
LFP91-3III	5/10	33.60±3.64♦*	50

♦p<0.05 compared with the NS group; *p<0.05 compared with the CTX group.

Extraction of Lentinula edodes C_{91-3} fermentation protein

Lentinula edodes C_{91-3} was inoculated in the sterile liquid medium containing 10 g/L glucose, 5 g/L yeast extract, peptone 1.0 g/L, vitamin B_1 0.01 g/L, vitamin B_2 0.01 g/L, and 1.0 g/L KH_2PO_4 in a 5 L bioreactor at 35°C, pH 6.5 at120 rpm, with air flow rate of 3 L/min for 7 days, and then continued fermenting at 25°C. When number of spores achieved 10^7/ml, the broth was collected and centrifuged for 10 min with 3,000 g/min at 4°C condition. The supernatant was collected and filtered through sterile filter paper (Size of filter). LFP_{91-3} from filtration liquid were gained through salting out with $(NH4)_2SO_4$ and then dialyzed with sterile distilled water. The protein samples were freeze dried and stored at 4°C till use.

Antitumor activity on S_{180} tumor-bearing mice *in vivo*

The S_{180} ascitic tumor cell number and viability were determined, and then cell concentration was adjusted to 1×10^7 cells per ml with complete RPMI 1640 medium. The inbred BALB/c mice were inoculated with 1 ml tumor cells by intra-peritoneal injection. After 24 h, we established S_{180} ascitic tumor cells randomly in mice (n=50) and divided the mice into five groups of 10 each: 1) normal saline (NS) control, 2) cyclophosphamide (CTX) group, 3) LFP_{91-3}I-treatment group, 4) LFP_{91-3}II- treatment group, and 5) LFP_{91-3} III-treatment group**.** The tumor-bearing in NS control group were injected intra-peritoneal with NS 1 ml, once daily for 5 days, CTX group to cyclophosphamide 0.4 mg, LFP_{91-3} Ⅰ group to LFP91 to 3 50 μg, LFP_{91-3} Ⅱ Group to LFP_{91-3} 100 μg; LFP_{91-3}Ⅲ group to LFP_{91-3} 150 μg. The dose, means, methods and frequency of injection were the same as the NS group. The growth rates and survival times of tumor-bearing mice were determined in the five groups. From these values, we calculated the viability of tumor-bearing mice that survived for a minimum of 60 days.

Antitumor test of LFP_{91-3} on S_{180} tumor cell through MTT (sigma) assay *in vitro*

The effect of LFP on S180 tumor cells was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Carmichael et al., 1987). S_{180} cells in the logarithmic phase were selected, and the cell survival rate was >95%, as determined by trypan blue staining. The cell concentration was adjusted to a final concentration of 5×10^6 cells per ml. and then 100 μl was added into 96-well plate per hole. After pre-incubating S_{180} tumor cells for 8 h at 37°C in 5% CO_2 incubator, the control group was added NS 100 μl. 100 μl of LFP_{91-3} (5, 10 and 15 μg/ml) with different concentrations was added as the experimental group and incubated with the cells for 24, 48 and 72 h. 20 μl of the MTT stock solution (5 mg/ml) was added to each well and incubated for 4 h, and the plate was centrifuged at 800×g for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl dimethylsulfoxide and then absorbance at 540 nm was measured on scanning multiwell spectrophotometer. The tumor inhibition ratio was calculated as follows:

Inhibition ratio = (1 - A540 in experimental group / A540 in control group) × 100%.

Apoptosis detection of LFP_{91-3} on S_{180} cell through flow cytometry *in vitro*

S_{180} cells in the logarithmic phase were selected, and the cell survival rate at >95%, was determined by trypan blue staining. The cell concentration was adjusted to a final concentration of 1×10^7 cells per ml. 100 μl cell suspension was added into 96-well plate per well. After pre-incubating S_{180} tumor cells for 8 h at 37°C in 5% CO_2 incubator, the control group contains normal saline 100 μl. 100 μl LFP_{91-3} with a final concentration (5 μg/ml) was added as the experimental group and incubated with the cells for 24, 48 and 72 h. The cells were collected and the cell concentration was adjusted to 5×10^5/ml. The cells were washed twice with PBS, centrifuged at 1000 rpm for 5 min and the supernatants were aspirated. The cells were suspended with 500 μl 1× Binding Buffer, followed by adding 5 μl Annexin V-FITC, 5 μl PI (BioVision Annexin V-FITC Apoptosis Detection Kit), keep in dark for 5min at room temperature, and then detected through flow cytometry.

Statistical analyses

The data were analyzed by ANOVA using SPSS software. Results are reported as mean ± standard deviation and $p<0.05$ was set as statistical significance.

RESULTS

Survival

The tumor visibly increased in size, and survival time decreased in the S_{180} tumor-bearing mice. We observed peritoneal swelling in the NS-control group by the seventh day after inoculation, and mortality was 100%, with an average survival time of 15.8 days (Table 1). In the CTX group, mortality was 100%, with an average survival time of 21.3 days (5.5 days longer than the control group; $p<0.05$). In the LFP_{91-3}I-treatment group, mortality was 80%, with an average survival time of 23.0 days (7.2 days longer than the control group; $p<0.05$). In

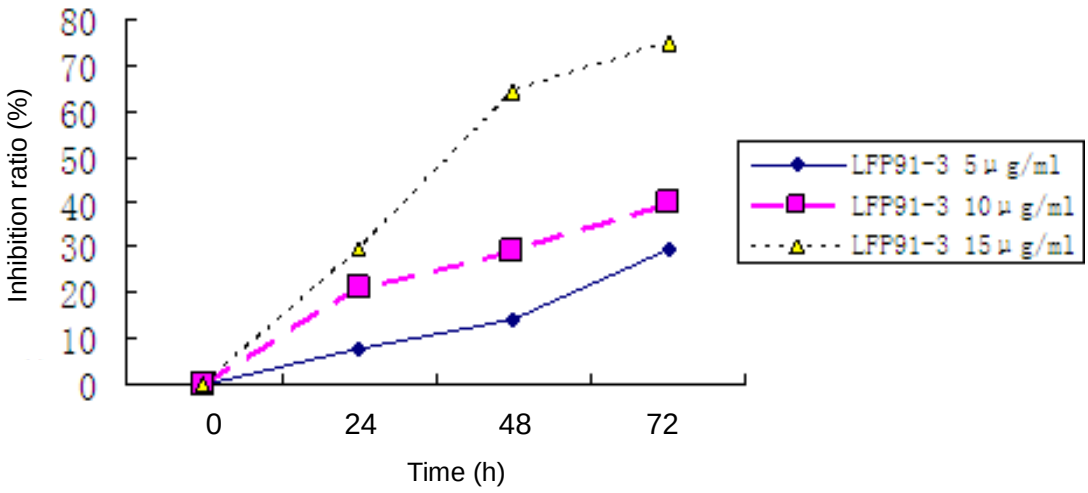


Figure 1. Inhibition ratio of S_{180} cell growth affected by different concentration LFP_{91-3}.

Table 2. Inhibition ratio of S_{180} cell growth affected by different concentration LFP_{91-3}.

Time (h)	Inhibition ratio (%)		
	5 μg/ml	10 μg/ml	15 μg/ml
24	8.16	21.42	29.89
48	14.45	29.17	64.14
72	29.91	39.90	74.53

Table 3. Apoptosis ratio of S_{180} in the initial stage between LFP_{91-3} experimental group and NS control group.

Time	Apoptosis ratio in the initial stage (%)		
	24 h	48 h	72 h
NS control group	0.82	0.93	1.22
LFP_{91-3} experimental group	2.56	7.51	8.47

the LFP_{91-3}II-treatment group, in contrast, mortality was only 60%, with an average survival time of 28.5 days (12.7 days longer than the control group; $p < 0.05$). In the LFP_{91-3}III-treatment group, mortality was 50%, with an average survival time of 33.6 days (17.8 days longer than the control group; $p < 0.05$). However, the survival time of tumor-bearing mice was depended the certain dose of LFP_{91-3}.

Growth-inhibiting effect on S_{180} cell line by LFP_{91-3}

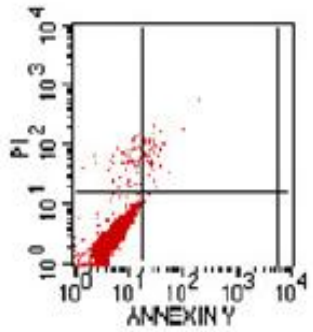
It can be seen from Figure 1 and Table 2, LFP_{91-3} could inhibit S_{180} cell growth directly, and its intensity and effectiveness depended on certain range of concentration and time.

Apoptosis analysis of S_{180} cell induced by LFP_{91-3}

The apoptosis with a significant increase in the initial stage effected by LFP_{91-3} (5 μg/ml) on S180 cells was 5.56% in 24 h, 7.51% in 48 h, 8.47% in 72 h (Table 3 and Figure 2D, E and F); and the apoptosis in NS control group was 0.82% in 24 h, 0.93% in 48 h, 1.22% in 72 h (Table 3, Figure 2A, B, and C). It was obviously that LFP91-3 experimental group was significantly higher than NS control group ($P<0.05$) Figure 3.

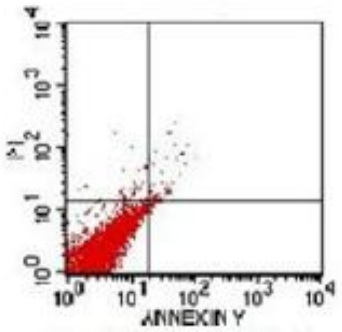
DISCUSSION

Each year, nearly 10 million new cases of malignant tumors occurs all over the world; moreover, 7 million died



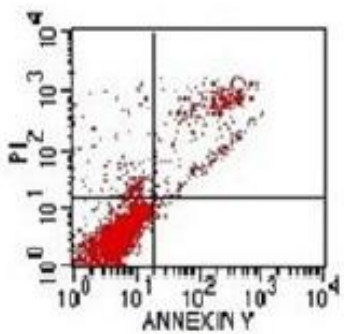
Quad	Events	% Gated
UL	170	1.96
UR	154	1.78
LL	8263	95.44
LR	71	0.82

2A : Apoptosis in NS control group in 24 h



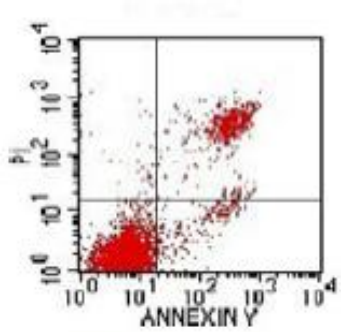
Quad	Events	% Gated
UL	94	1.01
UR	129	1.39
LL	9002	96.67
LR	87	0.93

2B Apoptosis in NS control group in 48 h



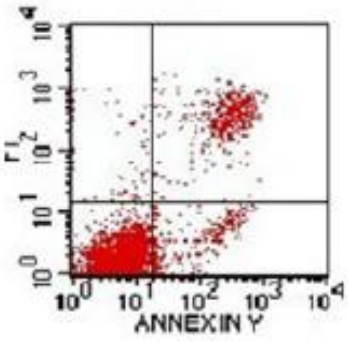
Quad	Events	% Gated
UL	640	7.95
UR	1044	12.96
LL	6273	77.88
LR	98	1.22

2C: Apoptosis in NS control group in 72h



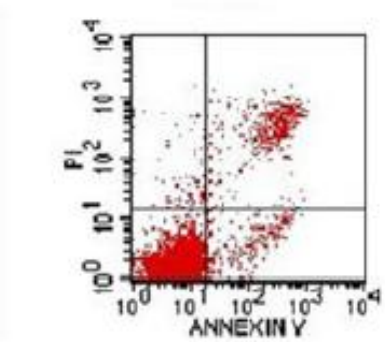
Quad	Events	% Gated
UL	160	1.74
UR	1582	17.17
LL	6931	75.23
LR	540	5.86

2D: Apoptosis in LFP_{91-3} experimental group in 24 h



Quad	Events	% Gated
UL	179	1.93
UR	1371	14.81
LL	7013	75.75
LR	695	7.51

2E: Apoptosis in LFP_{91-3} experimental group in 48 h



Quad	Events	% Gated
UL	226	2.36
UR	1453	15.16
LL	7092	74.01
LR	812	8.47

2F: Apoptosis in LFP_{91-3} experimental group in 72h

Figure 2. Apoptosis scatterplot of S_{180} in the initial stage between LFP_{91-3} experimental group and NS control group. (UL: upper left quadrant FITC-/PI+: impaired cell; UR: upper right quadrant FITC+/PI+, dead cell or non-viable apoptotic cell; LL: left lower quadrant FITC-/PI-, living cell: LR: lower right quadrant FITC+/PI-, apoptotic cell).

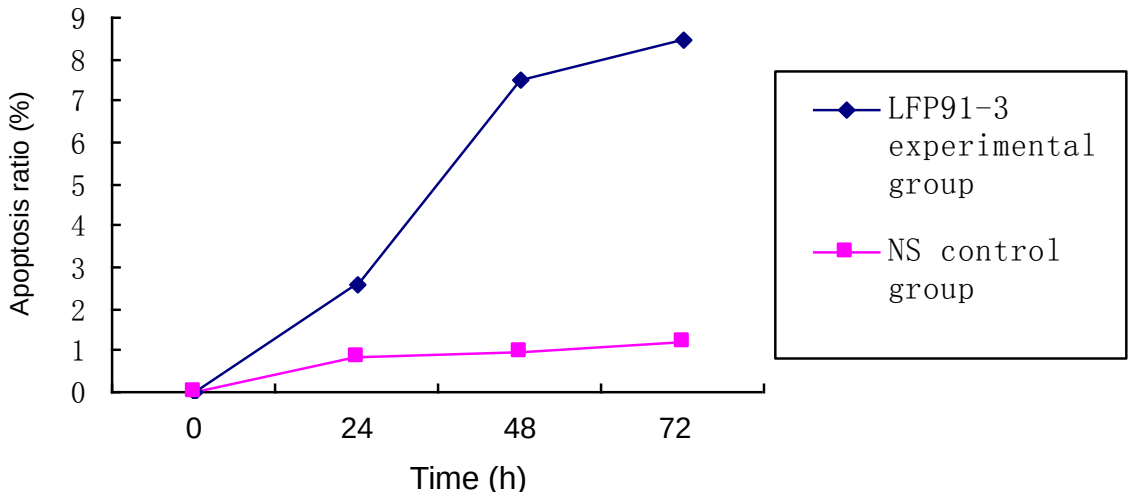


Figure 3. Apoptosis ratio of S_{180} in the initial stage between LFP_{91-3} experimental group and NS control group.

due to malignant tumors (Ebina, 2005). The studies on anti-tumor ingredients extracted from the fungus or its metabolites have increased researchers attention. Its antitumor activity has been recognized internationally. But there were many research have been done about polysaccharide extracts from Lentinula edodes (Borchers et al., 2004; Ooi and Liu, 2000; Kidd, 2005; Zheng et al., 2005; Fang et al., 2006; Miyaji et al., 2006; Ng and Yap, 2002). To explore its antitumor activity on protein level, we firstly found that the LFP_{91-3} gained through submerged fermentation could kill S_{180} tumor cells directly *in vitro*, and its mechanism related to apoptosis pathway induced by LFP_{91-3}. *In vivo,* LFP_{91-3} extended the life span of S_{180} tumor-bearing mice, but the mechanism needs to be further study. However, LFP_{91-3} was crude protein complexes. So further research will focus on the structure and biochemical character of protein monome and study the anti-tumor mechanism on apoptosis pathway in detail. In conclusion, our study provides a new kind of antitumor material and a new theoretical basis for further study of the mechanism in antitumor proteins from Lentinula edodes C_{91-3} Mycelia Fermentation Protein.

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