Immunomodulation and Anti-Cancer Activity of Polysaccharide-Protein Complexes

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Abstract: In the last three decades, numerous polysaccharides and polysaccharide-protein complexes have been isolated from mushrooms and used as a source of therapeutic agents. The most promising biopharmacological activities of these biopolymers are their immunomodulation and anti-cancer effects. They are mainly present as glucans with different types of glycosidic linkages such as (1 3), (1 6)-β-glucans and (1 3)-α-glucans, and as true herteroglycans, while others mostly bind to protein residues as polysaccharide-protein complexes. Three antitumor mushroom polysaccharides, i.e. lentinan, schizophyllan and protein-bound polysaccharide (PSK, Krestin), isolated respectively, from Lentinus edodes, Schizophyllum commune and Coriolus versicolor, have become large market items in Japan. Lentinan and schizophyllan are pure β-glucans, whereas PSK is a protein-bound β-glucan. A polysaccharide peptide (PSP), isolated from a strain of Coriolus versicolor in China, has also been widely used as an anti-cancer and immunomodulatory agent. Although the mechansim of their antitumor action is still not completely clear, these polysaccharides and polysaccharide-protein complexes are suggested to enhance cell-mediated immune responses in vivo and in vitro and act as biological response modifiers. Potentiation of the host defense system may result in the activation of many kinds of immune cells that are vitally important for the maintenance of homeostasis. Polysaccharides or polysaccharide-protein complexes are considered as multi-cytokine inducers that are able to induce gene expression of vaious immunomodulatory cytokines and cytokine receptors. Some interesting studies focus on investigation of the relationship between their structure and antitumor activity, elucidation of their antitumor mechanism at the molecular level, and improvement of their various biological activities by chemical modifications.

Introduction

Polysaccharides represent a structurally diverse class of macromolecules of relatively widespread occurrence in nature. Unlike proteins and nucleic acids, they contain repetitive structural features which are polymers of monosaccharide residues joined to each other by glycosidic linkages. Among these macromolecules, polysaccharides offer the highest capacity for carrying biological information because they have the greatest potential for structural variability. The nucleotides in nucleic acids and the amino acids in proteins can interconnect in only one way while the monosaccharide units in oligosaccharides and polysaccharides can interconnect at several points to form a wide variety of branched or linear structures [1]. The polysaccharide also forms secondary structures, depending on the conformation of sugar residues, the molecular mass, and the inter- and intra-chain hydrogen-bonding. For example, the number of possible permutations for four different sugar monomers can be up to 35,560 unique tetrasaccharides, whereas four amino acids can form only 24 different permutations [2]. This enormous potential variability in polysaccharide structure gives the necessary flexibility for the precise regulatory mechanisms of various cell-cell interactions in higher organisms.

In the last few decades, the biological activities of polysaccharides have attracted more and more attention in biochemistry and medicine. Chief among the most promising biopharmacological activities of polysaccharides are their immunomodulatory and antitumor effects. The earliest polysaccharide reported to have antitumor activity was isolated in 1943 from the bacterium *Serratia marcescens* and became known as Shear's polysaccharide [3]. Shear's polysaccharide which is actually a polysaccharide-lipid complex could cause extensive damage to Sarcoma 37 tumours in mice after intraperitoneal injection. However, as Shear's

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0929-8673/00 $19.00+.00

polysaccharide has undesirable toxic and other side effects, clinical tests have not been performed. Moreover, although many other polysaccharides from the bacteria such as *Escherichia coli, Streptococcus pyogenes* (OK-432), *Proteus vulgaris*, *Acetobacter xylinum* and *Salmonella typhimurium* have also been reported to exhibit cytotoxic effect against solid tumours [3], most of these bacterial polysaccharides belong to endotoxic lipopolysaccharides.

The search for potential polysaccharides as antitumor agents probably stems from dissatisfaction with cancer chemotherapy and radiotherapy. A large numbers of chemical compounds which have been identified as specific agents for killing cancer cells are also toxic to normal cells. Many of the potential anticancer drugs have considerable side effects and therefore have little clinical use. Hence, the discovery and identification of new safe drugs which are active against tumours becomes an important goal of research in biomedical sciences. The enhancement or potentiation of host defense mechanisms emerges as a possible means of inhibiting tumour growth without harming the host. Starting from this point of view, extensive studies have been made on polysaccharides extracted from microorganisms and plant sources.

Numerous antitumor polysaccharides have been discovered from mushrooms, fungi, yeasts, algae, lichens and plants [4-7]. This chapter will emphasise on the bioactive polysaccharides or polysaccharide-protein complexes from mushroom-type fungi which have been investigated with a view to their application as host defense potentiators or biological response modifiers for inhibiting tumour growth. The large mushroom-type fungi have a long history of medicinal usage in the Orient in addition to their nutritional value. Mushrooms are considered as macrofungi with a distinctive fruiting body which are large enough to be seen with the naked eyes. Most of macrofungi belong to the class Basidiomycetes but there are also others from class Ascomycetes [8]. The life cycles of mushroom-like fungi are complex and may involve a number of different morphological forms including mycelium and fruiting body stages.

β-D-Glucans, Heteroglycans and Polysaccharide-Protein Complexes

Antitumor polysaccharides have been reported to occur in the fruiting bodies, cultured mycelia and culture filtrates of basidiomycetes. These antitumor polysaccharides vary in their chemical composition, structure and antitumor activity [3,4,9,10]. Lucas *et al*. (1957) first reported the antitumor activity of extracts from the mushrooms, *Boletus edulis*, *Clitopilus abortivus* and others [11]. Since then, many antitumor polysaccharides were isolated from mushrooms and extensively studied, particularly in Japan [10,12-15]. Many polysaccharides (glycans) purified from mushroom fungi belong to either homoglycans or herteroglycans while others mostly bind to protein residues as polysaccharide-protein complexes. The fungal antitumor polysaccharides are mainly present as glucans with different types of glycosidic linkages, but some are true heteroglycans [16]. The main source of antitumor polysaccharides appears to be related to fungal cell walls which consist of polysaccharides such as chitin, cellulose, (1→3),(1→6)-β-glucans and (1→3)-α-glucans or polysaccharide-protein complexes such as galactomannan-protein, glucuromannan-protein [17]. However, chitin and chitosan (fungal chitin) have not been reported to have any antitumor activity [18].

In the seventies and eighties, three antitumor agents of polysaccharide nature i.e. lentinan, schizophyllan and protein-bound polysaccharide (PSK, Krestin), were isolated from *Lentinus edodes*, *Schizophyllum commune* and *Coriolus versicolor* respectively and have since become large market items in Japan [18]. Lentinan and schizophyllan are pure β-glucans [19-21] whereas PSK is a protein-bound β-glucan [22,23]. A polysaccharopeptide (PSP) has also been isolated from a strain of *Coriolus versicolor* in China and become widely used in clinical treatments as an anti-cancer and immunomodulatory agent [24]. PSP manufactured in China is found to be quite similar to PSK in Japan.

Lentinan from the fruiting body of *Lentinus edodes* is a representative mushroom (1→3)-β-glucan with effective antitumor and immunopotentiating activity. Its primary structure is a (1→3)-β-glucan consisting of five (1→3)-β-glucose residues in a linear linkage and two (1→6)-β-glucopyranoside branches in side chains which result in a right-handed triple helical structure [21]. Lentinan has a molecular weight of about 400-800x10^3.

Another highly potent antitumor polysaccharide, schizophyllan from *Schizophyllum commune* is also a (1→3)-β-glucan having a β-glucopyranosyl group linked 1→6 to every third or fourth residue of the main chain. It is similar to lentinan in its triple helix structure and biological activity, but physicochemically unlike lentinan. Its molecular weight is about 450x10^3 [19,25].

PSK (Krestin) prepared from *Coriolus versicolor* in Japan is different from lentinan and schizophyllan. It is a β-glucan-protein complex containing 25-38% protein residues. It has an average molecular weight of 94x10^3 as measured by ultracentrifuge analysis. It consists predominantly of acidic amino acids such as aspartic acid and glutamic acid and neutral amino acids such as valine and leucine but basic amino acids such as lysine and arginine are present only in small amounts. The major constituent monosaccharide is glucose with small

amounts of other sugar residues such as mannose, fucose, xylose and galactose. PSK is a (1→4)-β-glucan with (1→6)-β-glucopyranosidic side chains for every fourth glucose unit. It has a structure with branches at 3- and 6-positions in a proportion of one per every several residual groups of 1→4 bonds [22]. Polysaccharopeptide (MW 100K) isolated from a strain of *Coriolus versicolor* in China [24] is found to be quite similar in glucan structure to PSK in Japan. Two polysaccharide-protein complexes (PSPC), designated intramycelial PSPC (MW 28K) and extramycelial PSPC (MW 15K), have been isolated respectively from the cultured mycelia and culture filtrates of *Coriolus versicolor*, and a novel PSPC (MW15.5K) from cultured mycelia of *Tricholoma mongolicum* [26,27]. They activate both lymphocytes and macrophages from BALB/c mice and show no direct cytotoxic activity against fibroblasts, hepatoma cells and choriocarcinoma cells.

The antitumor polysaccharide extract from the culture filtrates of *Tricholoma lobayense* has been purified and characterized as a polysaccharide-protein complex (PSPC) which contains 54.3% polysaccharide consisting of galactose, glucose, arabinose, xylose, rhamnose, fucose and mannose, and 35.9% protein consisting mainly of aspartic acid, glutamic acid, serine, glycine, lysince and threonine [28,29]. The infrared spectra of PSPC indicates that PSPC possesses the characteristic groups of polysaccharide and intermolecular hydrogen bonds. The polysaccharide moiety of PSPC is an unique heteroglycan. PSPC inhibits the growth of Sarcoma 180 implanted in mice intraperitoneally or subcutaneously, with no sign of toxicity *in vivo*. The molecular weight of PSPC is estimated to be about $154x10^3$ [28]. Similarly, a protein-containing heteroglycan (MW 68K) has been extracted and purified from *Tricholoma giganteum* [30-32]. The composition of antitumor polysaccharide-protein comlexes from *Tricholoma* species are obviously different from those commercially available antitumor polysaccharides such as lentinan from the fruiting body of *Lentinus edodes* [20,21], schizophyllan from the culture filtrates of *Schizophyllum commune* [19,33], and PSK from the cultured mycelia of *Coriolus versicolor* [22]. The polysaccharide moiety of these three antitumor agents belongs to pure β-glucan.

Ganoderma lucidum and other related *Ganoderma* species are the most well known medicinal fungi in the Orient. Various antitumor polysaccharide components such as β- glucan, glucuronoglycan, mannoglucan and other active heteroglycans as well as polysaccharide-protein complexes have been extracted and purified for medicinal use [34-38]. Ganoderan (MW 20K), an immunomodulatory β-glucan of *G. lucidum*, induces potent antitumor immunity in tumour-bearing mice. It is mainly composed of glucose and 4% protein [38]. Several β-glucans, heteroglycans and glycan-protein complexes having high antitumor activity have been isolated from the fruiting bodies and mycelia of *Ganoderma applanatum*. The molecular weight of the primary polysaccharides ranging from $30x10^3$ to $1000x10^3$, and the basic chemical structure is (1→3)-β-glucopyranan having 1-15 (1→6)-monoglucosyl side chains [10]. It seems that the greater the molecular weight and the higher the water solubility of these polysaccharides, the higher the antitumor activity. Among the seven strong antitumor polysaccharide-protein complexes from *Ganoderma tsugae*, two were identified as protein-containing glucogalactans associated with mannose and fucose, and five were protein-containing (1→3)-β-glucans. It is noteworthy that antitumor polysaccharides having high activity from fruiting bodies were mostly heteropolysaccharides with molecular weight of about 10,000 and consisting of galactose, glucose, mannose and fucose, whereas the highly active polysaccharides from mycelia were mainly protein-containing glucans with molecular weight of 10,000 [18,39]. The active polysaccharides isolated from fruiting body and mycelium of these three *Ganoderma* species are markedly different in their component monosaccharides, their protein moiety content, and their average molecular weight.

Several potent antitumor polysaccharide-protein complexes have been purified from fruiting bodies of a Chinese edible mushroom, *Pleurotus sajor caju*. These include (a) protein-containing xyloglucan (MW 280K) with polysaccharide:protein ratio=76:24 (w/w); (b) protein-containing mannogalactan (MW 120K) with polysaccharide:protein ratio=76:16; (c) protein-containing xylan (MW 200K) with polysacchairde:protein ratio=62:21; (d) protein-containing glucoxylan (MW 90K) with polysaccharide:protein ratio=71:15; and (e) protein-containing xyloglucan (MW 70K) with polysaccharide:protein ratio=69:3 [40]. The polysaccharide with strong antitumor activity isolated from *Pleurotus ostreatus* is a highly branched (1→3)-β-glucan having an average structure represented by a pentasaccharide segment consisting of one nonreducing terminus, on 3,6-O-substituted, and three 3-mono-O-substituted β-glucopyranosyl side chains [41]. The more complete list of the antitumor polysaccharides and polysaccharide-protein complexes from mushroom fungi are presented separately in Table 1 and Table 2.

Antitumor Activities of Polysaccharide-Protein Complexes

In Vivo Studies

Numerous reports indicate that most of the polysaccharides or polysaccharide-protein complexes

from natural sources cannot exert any direct cytotoxic action on tumour cells. Their antitumor actions are predominantly considered to be host-mediated. It is possible that, in some instances, these two types of inhibitory action may be interwoven [3,42].

Many fungal polysaccharides or polysaccharide-protein complexes have distinct antitumor activities in murine allogeneic, syngeneic and autochthonous hosts [22,43,44]. The preliminary determination of antitumor activity has often relied on a bioassay system normally using Sarcoma 180 in mice based on an allogeneic tumour. There are a number of factors such as strain of mice, type of tumour, suitable dosage, and strictly planned timing of drug administration that are essential to achieve the antitumor effect of polysaccharides [3,21,45,46]. Hundreds of polysaccharides or polysaccharide-protein complexes have been screened for their antitumor activity, and three of them, namely schizophyllan, lentinan and protein-bound polysacharides (PSK and PSP), have been used clinically [21,24,47]. Schizophyllan (β-glucan) obtained from the culture filtrates of *Schizophyllum commune* inhibits solid Sarcoma 180 tumour when injected by intraperitoneal or intravenous route, but has low antitumor activity by subcutaneous route [19]. After schizophyllan is administered in mice by intraperitoneal or intravenous route, schizophyllan concentration in the serum as measured using sandwich ELISA method [48] falls gradually as time goes on [49]. Schizophyllan shows some interesting effects on the survival rate of mice intraperitoneally implanted with Sarcoma 180 ascites tumour cells. Whereas pretreatment with schizophyllan in the mice has no effect, combined pre- and post-treatment, and post-treatment, result in the increase of survival of 57% and 43% of the treated mice respectively. However, schizophyllan has no effect on the survival of mice intraperitoneally implanted with Sarcoma 37, Ehrlich carcinoma, or Yoshida sarcoma ascites tumors [3]. Lentinan (β-glucan) extracted from the fruiting body of *Lentinus edodes* exerts prominent antitumor activity against allogeneic, syngeneic, and autochthonous tumors in mice [43,50]. The antitumor activity of lentinan largely depends on the strains of mouse to which they are administered. A/J, DBA/2 and CD-1 mice are strongly responsive and complete tumor regression can be achieved in these strains, whereas it is somewhat less effective in BALB/c and CBA/J mice and ineffective in C3H/He and C57BL/6 mice [21]. When the DBA/2 and BALB/c mice are used, the intraperitoneal injection of lentinan inhibits the growth of methylcholanthrene-induced fibrosarcoma. Lentinan is also effective against various semisyngeneic tumors, such as P815 mastocytoma, L-5178Y lymphoma, virus-induced MM-46, and MM-102 carcinoma [21,51]. An alkaline-soluble (1→3)-β-glucan (MW 200K) prepared from *Flammulina velutipes* exhibits potent antitumor activity against Sarcoma 180 *in vivo* but not *in vitro* [52].

Protein-bound polysaccharide (PSK) prepared from the cultured mycelia of *Coriolus versicolor* in Japan exhibits a marked antitumor effect against allogeneic tumours such as Sarcoma 180 and Ehrlich carcinoma of experimental animals by both intraperitoneal and oral administration [22,47]. The blood radioactivity after oral administration of ^{14}C-labeled or ^{35}S-labeled PSK to the mice reaches maximal peak in 30-60 min and decreases thereafter. It was found that PSK is only partially decomposed to small molecules in the digestive tract. PSK is mainly absorbed in its large molecular form and exist in this state in the blood after administration of either ^{14}C-labeled or ^{35}S-labeled PSK. This suggests that ^{14}C-labeled or ^{35}S-labeled PSK is absorbed rapidly from the intestines and that the absorbed ^{14}C-labeled or ^{35}S-labeled PSK is transferred into the organs and then eliminated from the body [22]. When PSK is administered intraperitoneally at the time of subcutaneous inoculation of syngeneic tumours such as methylcholanthrene-induced fibrosarcoma in C57BL/6 mice, tumour growth is significantly inhibited [53] and *in vivo* tumour-induced angiogenesis is suppressed [54]. It is of interest that PSK shows some effects against semisyngeneic tumours such as adenocarcinoma 755 [55], mammary tumour S-MT [56], P388 leukemia and L1210 leukemia [54] under *in vivo* experimental conditions. As for rat tumours, PSK is effective against ascites hepatoma AH-13 by force feeding [54,57]. The survival period of rats bearing N-methyl-N-nitrosourea-induced mammary gland tumours is appreciably prolonged with PSK treatment [58]. Significant prolongation of disease-free period is gained by oral administration of PSK after curative surgical operation of colorectal cancer [59,60]. In China, similar polysaccharopeptide (PSP) isolated from the cultured mycelia of *Coriolus versicolor* also exhibits a wide range of strong antitumor activities, inhibiting Ehrlich ascites tumours, leukemia PS33, and monocytic leukemia [24]. A similar polysaccharide-protein complex [27,61] and a refined polysaccharide peptide fraction [62] from cultivated *Coriolus versicolor* significantly suppress the growth of tumour cells both *in vitro* and *in vivo*.

The antitumor polysaccharide-protein complex (PSPC) purified and characterized from the culture filtrates of *Tricholoma lobayense* exhibits strong antitumor activity in both ICR and BALB/c mice, with the most prominent effect in ICR mice [63]. In addition, the regression of transplanted Sarcoma 180 *in vivo* induced by PSPC in mice is evident as demonstrated using transmission electron microscopy [42]. PSPC has the ability to restore the phagocytic function of the peritoneal exudate cells (PEC) and the mitogenic

Table 1. Antitumor Polysaccharides From Mushrooms

No.	Scientific name	Active component	References
1	*Agaricus blazei*	Hetero-glycan (fruiting body)	141 142
2	*Agrocybe cylindracea*	α-Glucan (fruiting body)	112 120
3	*Amanita muscaria*	α-Glucan (fruiting body)	119 120
4	*Auricularia auricula*	β-Glucan (fruiting body)	143
5	*Crepidotus* sp.	Polysaccharide (medium product)	144
6	*Cryptoporus volvatus*	β-Glucan (fruiting body)	145
7	*Dictyophora indusiata*	β-Glucan (fruiting body)	146
8	*Flammulina velutipes*	Polysaccharide (β-glucan) (fruiting body)	52
9	*Ganoderma lucidum*	Polysaccharide (fruiting body) Ganoderan (β-glucan) (fruiting body, mycelium)	147 148 38
10	*Grifora frondosa*	β-Glucan (fruiting body, mycelium, medium product)	149 99
11	*Grifora umbellata*	Glucan (sclerotium, medium product)	13 150 114
12	*Hypsizigus marmoreus*	β-Glucan (fruiting body)	151
13	*Lampteromycer japonicus*	Mannan (fruiting body)	152
14	*Lentinus edodes*	Lentinan (β-glucan) (fruiting body, mycelium, medium product)	20 153
15	*Lepiota procera*	Polysaccharide (mycelium)	154
16	*Omphalia lapidescens*	β-Glucan (fruiting body)	116 115
17	*Pleurotus ostreatus*	β-Glucan (fruiting body)	155 41
18	*Polyporus confluens*	β-Glucopyranan (mycelium)	156
19	*Polyporus mylittae*	β-Glucan (fruiting body)	157
20	*Poria cocos*	β-Glucan (mycelium)	118
21	*Porodisculus pendulus*	β-Glucan (medium products)	114
22	*Sclerotinia libertiana*	β-Glucan (medium products)	114
23	*Schizophyllum commune*	Schizophyllan (β-glucan) (medium product)	19 114
24	*Trametes gibbosa*	Glucan (fruiting body)	158
25	*Tremella fuciformis*	Polysaccharide (fruiting body)	159
26	*Tricholoma giganteum*	Heteroglycan (fruiting body)	30
27	*Tylopilus felleus*	β-Glucan (fruiting body)	160
28	*Volvariella volvacea*	β-Glucan (fruiting body)	15

activity of T cells of tumour-bearing mice. PSPC also exhibits indirect cytotoxic activity against P815 mastocytoma cells and L929 mouse fibroblast cells by activating PEC to release reactive nitrogen intermediates (RNI) and tumour-necrosis factor-α (TNF-α) which are shown to increase significantly after PSPC treatment in the tumour-bearing mice [28,29]. RNI and TNF-α may critically affect the growth of P815 mastocytoma cells and L929 mouse fibroblast cells [64]. Similarly, the antitumor activity of ATOM, a polysaccharide-protein complex prepared from the cultured mycelia of *Agaricus blazei*, is highly effective against four kinds of established tumours, namely subcutaneously implanted Sarcoma 180 in mice Ehrlich ascites carcinoma, Shionogi carcinoma 42 and Meth A fibrosarcoma [65,66]. Heteroglycan-protein complexes

from *Grifola frondosa* are shown to depress tumour growth by activating the immune system as a biological response modifier [67,68]. Polysaccharides from other natural resources, for instance, curdlan from the bacterium *Alcaligenes faecalis* [69], funoran from the alga *Gloiopeltis tenax* (Ren *et al*. 1995) and acid polysaccharide from the plant *Pinus parviflora* [70] are found to have significant antitumor activity *in vivo*.

Some researchers use the conjugates of mitomycin C with antitumor polysaccharides such as schizophyllan [71] and PSK [72] to maintain the effect of the cancer chemotherapeutic agents and to reduce toxic side effects. The mechanism of the conjugates of antitumor polysaccharides with other chemotherapeutic agents including cyclophosphamide, nimustine, 5-fluorouracil [73,74], and carboquone on rat bladder carcinoma [75]

Table 2. Antitumor Polysaccharide-Protein Complexes From Mushrooms

No.	Scientific name	Active component	Reference
1	*Agaricus blazei*	β-Glucan-protein (fruiting body) β-Glucomannann-protein complex (ATOM) (mycelium)	109 66
2	*Agrocybe cylindracea*	Protein-bound polysaccharide (basidiocarp)	161
3	*Armillaria mellea*	Peptide-glucan (fruiting body)	162
4	*Armillariella tabescens*	Protein-containing heteroglycan (fruiting body)	163
5	*Collybia confluens*	Protein-bound polysaccharide (mycelium)	164
6	*Cordyceps ophioglossoides*	Protein-bound polysaccharide (medium product)	14 165
7	*Coriolus versicolor*	Protein-bound polysaccharide (mycelium)	22 26 166
8	*Flammulina velutipes*	Protein-bound glucan (fruiting body, mycelium)	167 168 58
9	*Fomes fomentarius*	Protein-containing polysaccharide (medium product)	169
10	*Fomitella fraxinea*	Protein-containing galacto-mannoglucan (fruiting body)	170 171
11	*Ganoderma tsugae*	Protein-containing glucogalactan (fruiting body) Glycan-protein complex (mycelium)	172 39
12	*Hericium erinaceum*	Protein-glycan complex (fruiting body)	10
13	*Laetiporus sulphureus*	Protein-polysaccharide (fruiting body)	173
14	*Phellinus linteus*	Carbohydrate-peptide (mycelium)Protein-bound polysaccharide	174 175 176
15	*Pleurotus citrinopileatus*	Protein-containing heteropolysaccharide (fruiting body)	177
16	*Pleurotus sajor caju*	Protein-containing xyloglucan, protein-containing mannogalatan, protein-containing glucoxylan (fruiting body)	40
17	*Polyporus confluens*	β-Glucopyranan-protein (fruiting body)	156
18	*Tremella fuciformis*	Heteroglycan-protein (fruiting body)	178 179
19	*Tricholoma giganteum*	Protein-containing polysaccharide (fruiting body)	30 31
20	*Tricholoma lobayense*	Polysaccharide-protein complex (PSPC) (medium product)	28 29 63
21	*Tricholoma mongolicum*	Polysaccharide-peptide complex (mycelium)	26

has been extensively studied. This kind of study provides possible means of improvement for more effective clinical applications of polysaccharides.

In Vitro Studies

Various glucans such as schizophyllan and lentinan show no direct growth-inhibitory effects on the tumour cell lines *in vitro*. [76-78] However, the indirect cytotoxicity of lentinan is observed *in vitro*. Lentinan enhances the antitumor cytotoxic activity of peritoneal macrophages against human melanoma target cells *in vitro* [79]. The activity of lymphokine-activated killer cells stimulated by IL-2 and lentinan against autologous tumour cells and K562 human erythroleukemia cells is greater than that stimulated by IL-2 alone *in vitro*. The optimal concentration of lentinan for the generation of killer cells ranges from 25 to 500 ng/ml, a level which could be achieved *in vivo* by the administration of clinical doses (2.0 mg/kg) of this agent [80,81]. Unlike most glucans, PSK (protein-bound polysaccharide) has both direct and indirect cytotoxic effects on tumour cell lines *in vitro*. When L1210 leukemia cells or P815 mastocytoma cells are mixed with PSK *in vitro*, and inoculated subcutaneously in BDF1 mice, tumour suppression takes place [22]. It is reported that PSK enhances the cytotoxic activity of peripheral blood lymphocytes (PBL) against T24 human urinary bladder tumour cell line after PBL are incubated with PSK at concentration of 10-100 μg/ml *in vitro* [82,83]. The level of DNA synthesis in PBL increases in the presence of PSK, and the maximum increase is obtained when PBL are cultured with PSK at 100 μg/ml for 5 days [84]. When L-929 cells are treated with TNF-α in the presence of 30 μg/ml of PSK *in vitro*, the cytotoxic action of TNF-α is enhanced [77,85]. Moreover, PSK inhibits growth and DNA synthesis *in vitro* in various cell lines such as L1210 leukemia, P388 leukemia [86], Ehrlich carcinoma, Yoshida sarcoma, AH-13 [22], human hepatoma C-HC-20 [87], human choriocarcinoma GCH-1 and GCH-2 [88], and human breast cancer cell MCF-7 [78]. These results show that antitumor effects of PSK may be, in part, due to its direct cytotoxicity. Similarly, PSP (polysaccharide peptide) has a wide range of antitumor activities *in vitro*, inhibiting Ehrlich ascites tumour, leukemia P388, Sarcoma 180 and four types of human cancer cell lines including human gastric cancer cells, human lung cancer cells, mononuclear leukemia cells and human skin histiocytic lymphoma cells [24]. In addition, a small polypeptide isolated from the crude PSP has higher cytotoxic effect than that of PSP and PSK on HL-60, LS174-T, SMMU-7721 and SCG-7901 cell lines [24]. PSK, PSP and most of β-D-glucans such as lentinan and schizophyllan are different in the mode of antitumor action *in vitro*. It is suggested that PSK and PSP may have some unique structural features, possibly generated from the involvement of protein portions and/or unique structural configurations including sugar to sugar linkages, that contribute to their immunomodulatory and antitumor actions [77].

Antitumor Mechanisms of Polysacchairde-Protein Complexes

Many polysaccharides or polysaccharide-protein complexes have been identified to have antitumor activities [3,5,18,21]. Although a complete answer to the antitumor mechanism of polysaccharides or polysaccharide-protein complexes is not yet available, they are generally considered as a kind of biological response modifiers which are able to restore or enhance various immune responses *in vivo* and *in vitro*. These macromolecules may have the immunotherapeutic property to inhibit growth of tumour cells and exert carcinostatic action, even though some of them may also possess direct cytotoxic effects on cancer cells.

Several polysaccharides such as lentinan (β-glucan) and PSK (β-glucan-protein) have been shown to have effective antitumor action against a variety of transplantable experimental animal tumours, and have been successfully used in clinical treatments [21,47]. Both cell-mediated immune response against the target cells initiated by macrophage-lymphocyte interactions and cytotoxicity induced by antibodies to target cells are believed to contribute to the elimination of target tumour cells [17,21]. Lentinan can restore and augment responsiveness of host cells, but has no direct cytotoxicity against tumours. The antitumor effect of lentinan is lost in the neonatal thymectomized mice, and decreased considerably by administration of antilymphocyte serum [89]. The results suggest that the antitumor action of lentinan requires an intact T-cell component and that the activity is mediated through thymus-dependent immune mechanism. Interestingly, the antitumor activity of lentinan is also inhibited by pretreatment with anti-macrophage agents. Thus, the various effects of lentinan are thought to be due to potentiation of the response of precursor T-cells and macrophages to cytokines produced by certain classes of lymphocytes after specific recognition of tumour cells [21]. In addition, the induction of a marked increase in the amounts of CSF, IL-1 and IL-3 by lentinan results in maturation, differentiation and proliferation of the immunocompetent cells for host-defense mechanism [21]. Similarly, lentinan is able to restore the suppressed activity of helper T-cells in the tumour-bearing host to their normal state, leading to complete restoration of humoral immune responses [90,91]. Moreover, it is reported that the delayed-type hypersensitivity response induced by lentinan are consistent with the antitumor activity of lentinan in the

tumour-bearing mice [92]. It is suggested that the delayed-type hypersensitivity response at the tumour sites induced by lentinan and the subsequent infiltration of immune effector cells, such as natural killer cells and cytotoxic T-lymphocytes, into the tumour burden are an important mechanism of antitumor action of lentinan. Recent observation shows that lentinan inhibits hepatic metastasis in adenocarcinoma 26−bearing mice by activated Kupffer cells [93]. However, it remains to be clarified which immunomodulatory effects induced by lentinan is a critical action for tumour rejection.

Schizophyllan is similar to lentinan in the composition and biological activity, and its mechanism of antitumor action appears to be quite similar [3,5]. The antitumor effect of schizophyllan is diminished in the mice neonatally thymectomized and treated with antithymus globulin. Schizophyllan restores and enhances cellular immunity in the tumour-bearing host by functioning as a T-cell adjuvant and macrophage activator [94,95]. In recent years, the induction of gene expression of cytokines by schizophyllan has been studied *in vitro* and *in vivo* [96-98]. After schizophyllan is administered intraperitoneally in ICR mice, the kinetics of gene expression of cytokines are different in peritoneal exudate cells, splenocytes and liver cells. It is generally accepted that protein synthesis and gene expression of cytokines are regulated separately. Therefore, the antitumor activity of schizophyllan is mainly due to host-mediated immune responses [97,98]. Another (1→3)-β-glucan, grifolan from *Grifola frondosa,* is similar to schizophyllan in the primary structure [99]. Enhancement of the mRNA levels of IL-6, IL-1 and TNF-α of macrophages by grifolan treatment is detected *in vitro* by reverse transcription-polymerase chain reaction (RT-PCR), showing that grifolan is a novel macrophage activator which increases cytokine production [100].

PSK has no substantial effect on immune responses of the host under normal conditions [22]. It has the ability to restore immune potential to the normal level after the host has been depressed by tumour-burden or anticancer chemotherapeutic agents [22,47,73,101]. In ICR mice, antibody production against trinitrophenyl that has depressed the immunity in Sarcoma 180-bearing mice can be restored by PSK administration. In AKR mice, on the other hand, antibody production is not decreased in Sarcoma 180-bearing mice and is not augmented by PSK [45]. Oral administration of PSK can improve the impaired antitumor $CD4^+$ T-cell response in gut-associated lymphoid tissue of specific-pathogen-free mice [102]. PSK enhances the cytotoxic activity of peripheral blood lymphocytes (PBL) *in vivo* and *in vitro*. It may accelerate interaction of PBL with tumour cells such as T24 human urinary bladder tumours when both effector cells and target cells are exposed to PSK simultaneously. Moreover, after the intratumoral administration of PSK, it may come into close contact with tumour cells, whereupon local inflammatory responses occur and result in the non-specific killing of tumour cells [74]. Therefore, local administration of PSK is more efficient than systemic one [82]. It is reported that PSK induces gene expression of some cytokines such as TNF-α, IL-1, IL-8 and IL-6, *in vivo* or *in vitro* [42,103-105]. These cytokines produced by monocyte, macrophage and various other cell types mediate multiple biological effects by direct stimulation of cytotoxic T-cells against tumours, enhancement of antibody production by B-lymphocytes and induction of IL-2 receptor expression on T-lymphocyte. The induction of TNF-α by PSK would contribute, in part, to potent tumoricidal effects of this agent since the administration of neutralizing antibody against TNF-α significantly attenuates the antitumor activity of PSK in the murine model [105]. The recent study also indicates that PSK exerts tumoricidal activity by inducing T-cells that recognize PSK as an antigen and kill tumour cells in an antigen-specific manner [106]. Similarly, polysaccharide peptide (PSP) is able to suppress the growth of various human cancer cell lines and reverse tumour-induced immunodeficiencies in mice by increasing IgG and C3 complement levels [24,107].

Moreover, many polysaccharides or polysaccharide-protein complexes also exhibits antimetastatic effects. Lentinan not only markedly prevents chemical and viral carcinogenesis [43], but also suppresses cancer metastasis and recurrence in animal models [108]. PSK has also been shown that, once the progression of carcinogenesis is initiated, it exhibits significant preventive action on cancer metastasis such as the suppression of pulmonary metastasis of methylcholanthrene-induced sarcomas, human prostate cancer DU145M, and lymphatic metastasis of mouse leukemia P388 in the spontaneous metastatic models, as well as the inhibition of metastasis of rat hepatoma AH60C, mouse colon cancer 26, and mouse leukemia RL male 1 in the artificial metastatic models [23]. It is reported that PSK is able to influence the following steps of cancer metastasis: (a) through the inhibition of tumor invasion, adhesion and production of cell matrix-degrading enzymes; (b) by suppression of tumor cell attachment to endothelial cells; (c) by suppression of tumor cell motility and thus cell migration after extravasation; (d) through the inhibition of tumor angiogenesis and growth; (e) through the modulation of cytokine production and the augmentation of effector cell functions; and (f) by suppression of malignant progression of tumour cells through superoxide trapping [23]. The induction of immunomodulatory cytokines in peripheral blood mononuclear cells by orally administered PSK may

account, in part, for the multiple immunomodulating activities *in vivo* [105]. The antitumor mechanism of lentinan and schizophyllan has been demonstrated to involve the activation of the host immune system, through the regulation of cytokines in the cytokine network and activation of complement system [96]. Cytokines which are antigen-nonspecific protein or glycoprotein, are synthesized and generally rapidly secreted by cells in response to a stimulus. They are believed to act over a short range and to have very short half-lives. The induction of gene expression of nine out of seventeen cytokines and five out of six cytokine receptors in peritoneal exudate cells and splenocytes by administration of PSPC prepared from *Tricholoma lobayense* is confirmed using reverse transcription-polymerase chain reaction (RT-PCR) and dot-blot hybridization [42]. This suggests that the immune cells are responsive to PSPC in terms of gene expression and production of immunomodulatory cytokines which might mediate the immunopotentiation of this agent *in vivo*.

β-Glucans (e.g. lentinan) and protein-containing glucans or heteroglucans (e.g. PSK and PSP) are different in their chemical composition and biological activity. It remains to be elucidated whether the differences of their biological activity are due to the presence of protein residues bound to the polysaccharide as complexes.

Structure and Antitumor Activities of Polysaccharides

Polysaccharides having antitumor action differ greatly in their chemical composition and configuration and physical properties. Antitumor activity is exhibited in a wide range of glycans extending from homopolymers to highly complex heteropolymers. Although it is difficult to correlate the structure and antitumor activity of complex polysaccharides, some possible relationships can be inferred. It has been reported that most of the antitumor polysaccharides such as lentinan and schizophyllan show the same basic β-glucan structure with different types of glycosidic linkages. Therefore it is obvious that some structural features such as β-1,3-linkages in the main chain of the glucan and further β-1,6-branch points are needed for antitumor action [4]. The β-glucans containing mainly 1,6-linkages have less activities. Glucans with high molecular weight appear to be more effective than those with low molecular weight [5,77]. However, obvious variations of antitumor polysaccharides are also noted. There are antitumor polysaccharides with other chemical structures, such as hetero-β-glucan [18], heteroglycan [32], β-glucan-protein [109], α-manno-β-glucan [18], α-glucan [110], α-glucan-protein [18], and heteroglycan-protein complexes [31,40,42]. For example, PSK and PSP are a β-glucan-protein whereas PSPC from *Tricholoma* species are a heteroglycan-protein complex [31,42]. Therefore, it is difficult to identify which polysaccharide structure is essential for antitumor action. It has been shown that the molecular mass, the degree of branching, conformation and chemical modification of antitumor polysaccharides significantly affect their antitumor and immunomodulatory activities [21,25,99,111,112].

The Effect of Molecular Mass

When PSK is separated into four fractions with different molecular masses (F1:<50 KDa; F2: 50-100 KDa; F3: 100-200 KDa; F4:>200 KDa) by successive filtration, the highest molecular mass fraction (>200 KDa) has the most potent immunomodulatory activity [76,85]. A (1→3)-β-glucan prepared from the cultured mycelium of *Grifola frondosa* manifests changes of biological activities with various molecular masses obtained by heat treatment for varying times at 150°C [99]. The highest molecular mass fraction (800 KDa) always exhibits most strong antitumor and immunomodulatory activities [85,99,113]. These investigations raise the possibility that antitumor polysaccharides may not always be multiple enhancers of the host defense system, and that high molecular mass is required for extensive enhancement of immunological and antitumor activities. On the contrary, lentinan and schizophyllan with low molecular weight exhibit the same antitumor activity against Sarcoma 180 as those with higher molecular weight [33,80,114]. The discrepancy remains to be clarified.

The Impact of Branching Configuration

In general, β-glucans are active antitumor agents if they are mainly linear, possessing not excessively long branches. For example, pachyman, a branched β-glucans obtained from *Poria cocos* is inactive, whereas pachymaran, obtained by debranching pachyman using periodate oxidation and mild hydrolysis, exhibits pronounced antitumor activity [111]. Lentinan (2/5) is a β-1,3-D-glucan with two branches for every five D-glucopyranosyl residues [21]. Schizophyllan (1/3) is also a β-1,3-D-glucan with one branch for every three D-glucopyranosyl residues [33]. The moiety of polysaccharide of PSK (1/5) is a (1-3)-β, (1-4)-D-glucan of one branch for every five D-glucopyranosyl residues [22]. Although the degree of their branches is different, their antitumor activities are not obviously different. However, the debranched lentinan preparations are more effective against Sarcoma 180 than the native lentinan at a dose of 2.0 mg/kg for five days in mice [80]. In addition, a highly branched β-1,3-

D-glucan (2/3, two branches for every three D-glucopyranosyl residues), called OL-2, extracted from *Omphalia lapidescens* also shows antitumor activity on the solid form of Sarcoma 180 when administered intralesionally, and increases the life span of mice treated with ascites form of Sarcoma 180 and MH 134 hepatoma [115,116]. At the molecular level, it is found that the cytokine expression patterns between schizophyllan (1/3) and OL-2 (2/3) of antitumor polysaccharides are also different [96,97]. The OL-2 administered mice strongly express IL-1α, IL-1β and IL-1ra genes in peritoneal exudate cells, while schizophyllan only induces the expression of IL-1α. In the genes related to haematopoiesis, OL-2 induces the expression of G-CSF and GM-CSF, but schizophyllan induces the expression of IL-3 [96,97]. Therefore, the relationships between antitumor activity and the branch ratios of β-glucan are quite complicated.

The Relationship Between Antitumor Activity and Conformation

Conformations of antitumor polysaccharides include single helix, triple helix and random coiled conformers. A triple helix conformer is usually more stable than the single helix conformer, since a certain part of the single helix conformer is gradually changed to the triple one. Lentinan, schizophyllan and PSK all have triple helix structure [21,22,25]. Pachyman from *Poria cocos* which is a β-1,3-glucan and has a single helix conformer is biologically inactive against tumor growth. However, when pachyman is treated with periodate oxidation and mild hydrolysis, the newly formed conformer, pachymaran or carboxyl-methyl-pachymaran exhibits pronounced antitumor activity [111,117,118]. Schizophyllan-OH which has a single helix structure derived from the alkaline-treated schizophyllan shows a reduced ability to inhibit tumour growth as compared to the native schizophyllan [21]. Therefore, the antitumor activity of polysaccharide apparently depends on the helical conformation [25]. The relationship of conformation and antitumor activity of polysaccharides or polysaccharide-protein complexes suggests that the existence of biological system within the host body recognizes the configurational structure of polysaccharide.

Improvement of Antitumor Activity by Chemical Modification

To improve the biological activity of antitumor polysaccharides by chemical modification, carboxymethylated (CM), hydroxylated, formylmethylated, aminethylated and sulfated products have been designed. The linear (1→3)-α-glucans from *Amanitamuscaria* and *Agrocybe cylindracea* have little antitumor activity [112,119]. However, the carboxymethylated linear (1→3)-α-glucan shows high potent antitumor activity against Sarcoma 180 and immunomodulating activity in mice [120]. Debranched pachymaran and CM-pachynmaran is a more effective antitumor β-1,3-D-glucan [111,117,118]. The nitric oxide and TNF-α concentrations from peritoneal exudate cells induced by hydroxylated schizophyllan administration are higher than those by native schizophyllan *in vivo* [25]. The antitumor activity of the formylmethylated and aminoethylated derivatives of schizophyllan against Sarcoma 180 solid tumour in mice is increased more effectively than that of the native schizophyllan [121]. It is noteworthy that the sulfated lentinan and schizophyllan products exhibit strong anti-human immunodeficiency virus activity though with reduced antitumor effect [122-124]. These studies show that the chemical modification of polysaccharides might be an effective approach of improving the biological activities of polysaccharides.

Other Biological Activites

Antimicrobial Activity

Antimicrobial drugs have been employed for prophylactic and therapeutic purposes. However, recent occurrence of drug-resistant strains has made treatment difficult. Thus, the antimicrobial activity of various antitumor polysaccharides is evaluated in term of their clinical efficacy. Schizophyllan is reported to have the ability to enhance protection against *Streptococcus* sp. infection [125]. Lentinan is therapeutically effective against *Mycobacterium tuberculosis* and *Listeria monocytogenes* [21]. PSK induces potent antimicrobial activity against *Escherichia coli*, *Listeria monocytogenes* and *Candida* [22,77]. The acidic polysaccharides from the plant *Pinus parviflora* exhibits antimicrobial activity against *Escherichia coli* [126]. It is suggested that the phagocyte activation and reactive nitric oxide (NO) production of macrophages contribute to the antimicrobial activity of polysaccharides [22,127].

Antiviral Activity

PSK has been shown to possess antiviral activities against ectromelia virus and cytomegalovirus infections [22] and cell-free infection of human immunodeficiency virus (HIV) [128]. It is reported that the degraded form of schizophyllan [129] and the extracts from *Ganoderma lucidum* [130] also have anti-HIV activity. Moreover, lentinan is able to inhibit replication of adenovirus type 12, Abelson virus and VSV-encephalitis virus [21]. Although lentinan itself has no ability to block HIV infection, concomitant treatment with 3'-azido-3'-deoxythymidine (AZT) suppresses the

surface expression of HIV antigens more than does AZT alone. It is possible that lentinan augments AZT efficacy in inhibiting HIV replication through stimulation of various cell lines to produce a variety of factors that interfere with HIV replication [128]. AZT generally has toxic side effects in AIDS patients. Thus, the modification of antitumor polysaccharides especially attracts attention. Sulfated lentinan and sulfated schizophyllan have strong anti-HIV activities though their antitumor activity is decreased or abolished [122-124]. However, it is still not clear how sulfated lentinan and sulfated schizophyllan exert their inhibitory action against HIV. It appears that lentinan are able to potentiate the physiological constitution of the host and enhance host resistance against infections from various types of bacteria, viruses, fungi and parasites [131].

Antioxidant and Free Radical Scavenging Activities

Reactive oxygen species produced by sunlight, ultraviolet, ionizing radiation, chemical reactions and metabolic processes, have a wide variety of pathological effects, such as DNA damage, carcinogenesis and cellular degeneration related to aging [132]. Antioxidant and free radical scavenging activities of natural products obviously can counteract these injurious and carcinogenic changes [133,134]. The extracts of *Ganoderma lucidum* improve endurance and antifatigue ability of the host under the condition of lack of oxygen. It is known that the extracts of *Ganoderma lucidum* can strongly remove the hyperoxide radical which is thought to be one of the main factors in ageing process of the body [135,136]. Superoxide radical could be quenched rapidly in the presence of PSK in the cell-free system consisting of hypoxanthine-xanthine oxidase, whereas natural glucans, such as schizophyllan, could not [77]. It is shown that PSK expresses the mimetic activity of superoxide dismutase (SOD) and promotes oxidative stress relief for cancer-bearing hosts [137,138]. PSK protects macrophages from lipoperoxide accumulation and foam cell formation caused by oxidatively modified low-density lipoprotein [139]. PSP is also considered as a good antioxidant as it exhibits strong scavenging effects on superoxide and hydroxyl radicals [140]. In a recent study, six out of eight mushroom antitumor polysaccharide preparations show significant superoxide and hydroxyl radical scavenging activities [136].

Conclusions

In the last few decades, large number of mushroom fungi have been increasingly used as a source of medicinal compounds and therapeutic adjuvants or health food supplements. Recently, the biological activities of polysaccharides or polysaccharide-protein complexes derived from mushrooms have received much attention in biomedical sciences. One of the most promising activities of these polysaccharides is their immunomodulation and anti-cancer effects. However, the mechanism of antitumor actions of polysaccharides or polysaccharide-protein complexes isolated from most fungi is still not completely understood. It is widely accepted that antitumor polysaccharides from higher fungi enhance various immune responses *in vivo* and *in vitro,* and act as biological response modifiers [21,22]. It is suggested that the cell-mediated immune response plays a critical role in antitumor activity of polysaccharides or polysaccharide-protein complexes [92]. Potentiation of the host defence system may result in the activation of many kinds of immune cells that are vitally important for the maintenance of homeostasis. Various experimental evidences support that the antitumor action of mushroom polysaccharides or polysaccharide-protein complexes is due to the enhancement and potentiation of cell-mediated immune system through the regulation of immunomodulatory cytokines and activation of complement system. Polysaccharides or polysaccharide-protein complexes are considered as multi-cytokine inducers that are able to induce gene expression of various cytokines and cytokine receptors [42,105]. A thorough understanding of immunomodulatory actions of polysaccharides or polysaccharide-protein complexes at the cellular and molecular levels will help elucidate their antitumor mechanisms. Future studies should therefore focus on investigation of the relationship between their structure and antitumor activity, elucidation of their antitumor mechanism at the molecular level, and improvement of their various biological activities by chemical modifications. New potential host defence potentiators with novel therapeutic characteristics from natural sources should be further developed.

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