CHAPTER 25

DIVERSE BIOLOGICAL ACTIVITY OF PSK (KRESTIN), A PROTEIN-BOUND POLYSACCHARIDE FROM *CORIOLUS VERSICOLOR* (FR.) QUEL.

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1. INTRODUCTION

PSK (trade name, Krestin) is a protein-bound polysaccharide, extracted by hot water from the mycelia of *Coriolus versicolor* (Fr.) Quel. (Kawaratake). Kureha Chemical Industry Company Limited paid attention to the effectiveness of oral administration of Polyporaceae (one of the Basidiomycetes) on stomach cancer patients. This company screened over 200 species of the fruit bodies of the Basidiomycetes for their antitumor activity against various tumor cells, including sarcoma-180, and found several promising Polyporaceae strains. Among these strains, *Coriolus versicolor* (Fr.) Quel. was considered to be the most suitable for further fractionation due to its high antitumor activity and stability during serial cultivation. Extracts of cultured mycelia of *Coriolus versicolor* had antitumor activity comparable to that of the fruit body. In 1971, the active principle was precipitated from extracts of cultured hyphae of *Coriolus versicolor* (Fr.) Quel (CM-101 strain) with saturated ammonium sulfate, desalted and named PSK (Hirose, 1988). PSK has been reported to induce host-mediated antitumor activity (Tsukagoshi *et al.*, 1984). Clinical application of PSK to cancer patients is only permitted in combination with other chemotherapeutic agents.

PSK has been shown to contain at least four different subfractions which are separable by stepwise filtration through a membrane filter (Tsuchitani *et al.*, 1987) or by isoelectric precipitation (Hirose 1988). However, most previous PSK studies have been done using unfractionated samples. This review summarizes data from our groups and others on the biological activity of unfractionated PSK (section 1-3) and fractionated PSK (section 4). Unique biological activities of PSK, in comparison with other glucans, are listed in section 5.

2. CHEMICAL PROPERTIES

PSK is a brownish powder comprised of protein (38%) and polysaccharide. It is highly soluble

in water but only slightly soluble in most organic solvents (methanol, pyridine, chloroform, benzene, hexane). The mean molecular weight of PSK, determined by ultracentrifugation, is 94 kD. The main component of the carbohydrate moiety is glucose, with galactose, mannose, xylose and fucose as minor components. The protein moiety is rich in acidic amino acids (such as aspartic acid, glutamic acid etc.) and neutral amino acids (such as valine and leucine etc.), with basic amino acids (such as lysine, arginine etc.) in lower amounts (Hirose, 1988).

The main component unit of the polysaccharide moiety of PSK is assumed to be a β-glucan, judging from specific rotation and cellulase digestion. Oxidation by periodic acid, Smith degradation and methylation analysis have suggested that the main chain consists of β-(1-4) glucose polymer, branched at positions 3 and 6 of the glucose.

PSK seems to have two forms of binding between the protein and polysaccharide regions: one is O-glycoside bonding between a serine or a threonine residue in the peptide chain and an OH-group in the sugar chain; and the other is N-glucoside bonding between an aspartic acid and an OH-group.

3. INDUCTION OF ANTIMICROBIAL ACTIVITY *IN VIVO*

3.1. Antimicrobial Spectrum

PSK induced potent antimicrobial activity against *Escherichia coli* in ICR mice (Sakagami, *et al.*, 1990d). Significant antimicrobial activity was induced when the mice were pretreated with 40 mg/kg PSK by an intraperitoneal, subcutaneous or intramuscular route, but not by an intravenous route, 48 h before *E. coli* inoculation. Intraperitoneal administration of PSK was most potent. Repeated oral administration of PSK for 2 weeks before *E. coli* inoculation was also effective (Sakagami *et al.*, 1990d). It has been reported that orally administered ^{14}C-PSK was absorbed through the digestive tract, and both low and high molecular weight substances appeared in the blood (Fujita, 1988). Undegraded PSK and PSK-derived low molecular weight substances were distributed to various organs (Fujita, 1988).

PSK induced potent antimicrobial activity against other micro-organisms such as *Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans, Klebsiella pneumoniae* (Mayer & Drews, 1980), *Mycobacterium leprae, Listeria monocytogenes, Serratia marcescens, Streptococcus pneumoniae, Bacteroides fragilis, Cryptococcus neoformans* and *Aspergillus fumigatus*.

3.2. Mechanism

Three possible mechanisms of antimicrobial activity induction by PSK will be considered: First, the induction of antimicrobial activity might be mediated via direct bactericidal activity of the induced polymorphonuclear cells (PMN). Administration of PSK rapidly induced the intraperitoneal accumulation of PMN, which generated higher amounts of luminol-dependent chemiluminescence (LDCL)(Sakagami *et al.*, 1990d). Although PSK administration stimulated the LDCL generation by peritoneal macrophages, the absolute value of LDCL generated by macrophages was only 5% of that of PMN (Harada *et al.*, 1989). Therefore, the major cell population, which is responsible for induction of the antimicrobial activity, seems to be PMN.

Second, the antimicrobial activity of PSK may arise from its ability to induce antimicrobial cytokines, such as tumor necrosis factor (TNF)(Nakane, 1988) and interleukin-1 (IL-1)(Ozaki *et al.*, 1987). Intravenous administration of PSK stimulated the endogenous production of TNF and IL-1a

in mouse serum (Sakagami, 1990a; Sakagami & Takeda, 1992c). The amount of TNF induced in the blood was significantly increased when the mice were treated several hours later with bacterial preparations, such as OK-432 (Picibanil)(Sakagami, 1990c) or heat-killed *Lactobacillus casei* (Sakagami, 1992b). The priming activity of PSK was slightly greater than that of other antitumor polysaccharides (Sakagami *et al.*, 1990c).

Third, the antimicrobial activity of PSK might come from its direct activation of myeloperoxidase (MPO)-halide antibacterial systems described below.

4. *IN VITRO* EFFECTS

4.1. Stimulation of Monocytes/Macrophages

When mouse macrophages were cultured with 100 ug/ml PSK, they became enlarged and elongated (Kamisato & Nowakowski, 1988). The morphological changes were associated with an increase in NBT-reducing activity and TNF production (Kurakata *et al.*, 1991). PSK stimulated the production of IL-1-like activity, assayed with the thymocyte proliferation assay (Kurakata *et al.*, 1991). In contrast, natural glucans [native Schizophyllan (MW 6000 kD), lower MW Schizophyllan (MW 450 kD)] and chemically modified glucans [CM-TAK (MW 97 kD), paramylon sulfate (MW 151 kD)] failed to stimulate morphological maturation, regardless of their molecular weights (Kurakata *et al.*, 1991).

PSK stimulated the production of IL-1 by human peripheral blood mononuclear cells more efficiently than the production of TNF (Hirose *et al.*, 1990; Sakagami *et al.*, 1993b) The ability of PSK to stimulate TNF or IL-1 production by mononuclear cells was more potent than that of other natural products such as lignified materials and tannin-related compounds (Utsumi *et al.*, 1993; Sakagami *et al.*, 1993b). More IL-1α was accumulated in the cells than in the medium fraction after PSK treatment, whereas IL-1β was distributed evenly into both fractions. PSK stimulated the production of adherent mononuclear cells, in which significantly more IL-1α/Il-1β was accumulated per cell than in nonadherent cells. Although IL-1α mRNA synthesis (assessed by a Reverse Transcriptase-Polymerase Chain Reaction) was slightly enhanced, IL-1β mRNA synthesis was not significantly changed by PSK treatment. This suggests that PSK might increase the efficiency of IL-1 mRNA translation or post-translational processing of IL-1 protein (Sakagami *et al.*, 1993a). Despite potent cytokine-inducing activity, lipopolysaccharide (LPS) did not significantly stimulate the production of adherent cells. These data suggest that PSK and LPS might stimulate mononuclear cells by different mechanisms.

4.2. Stimulation of PMN and Monocyte Iodination

PSK stimulated the iodination (incorporation of radioactive iodine into acid-insoluble fraction) (Klebanoff & Clark, 1977) of human peripheral blood PMN, monocytes and human promyelocytic leukemic cells of the HL-60 cell line, which had higher amounts of MPO (Ackerman & Douglas, 1986; Bainton, 1981). The PSK stimulation of iodination of both PMN and HL-60 cells was significantly suppressed by the presence of MPO inhibitors (Sakagami *et al.*, 1990b). This indicates that the stimulation of iodination by PSK depends on the presence of MPO. The products of MPO reaction (such as hypochlorite), which might be highly antibactericidal, remain to be investigated. In contrast, various glucans such as native (Schizophyllan) and chemically modified glucans

(CM-TAK, paramylon sulfate) did not significantly stimulate the iodination of either PMN or HL-60 cells (Sakagami *et al.*,1990b).

4.3. Stimulation of Cytokine Action

PSK stimulated the action of TNF in various assay systems. TNF has been reported to induce monocytoid differentiation of human myelogenous leukemic cells (Takeda *et al.*, 1986; Weinberg & Larrick, 1987). PSK stimulated the differentiation of human myeloblastic leukemic ML-1 cells induced by macrophage conditioned medium or 10 ng/ml TNF (Sakagami *et al.*, 1989). The extent of differentiation was assayed by NBT-reducing activity and expression of α-napthyl acetate esterase activity. PSK stimulated the cytotoxic activity of PSK against L-929 cells (Sakagami *et al.*, 1991a). It has been reported that TNF is a weak direct stimulant of the PMN respiratory burst and degranulation, as measured by iodination, H_2O_2 and lysozyme release (Klebanoff *et al.*, 1986). PSK further stimulated the iodination, in either an additive or a synergistic manner, whereas human natural interferon-γ (IFN-γ) or recombinant GM-CSF was not stimulatory (Sakagami *et al.*, 1990b).

PSK stimulated the action of IFN-γ. IFN-γ has been reported to induce monocytoid differentiation of the cells of various myelogenous leukemic cell lines, but the differentiation-inducing ability of IFN-γ varies considerably with different target cell lines (Ball *et al.*, 1984; Kim *et al.*, 1990). Upon incubation with IFN-γ, human histiocytic lymphoma U-937 cells produced more differentiated cells, whereas human monoblastic leukemic THP-1 cells produced fewer differentiating cells. PSK further potentiated the differentiation of both of these cells beyond that induced by IFN-γ alone (Kim *et al.*, 1990).

Either increase of cytokine binding to the cellular receptor, or inhibition of receptor down regulation, has been suggested to be a possible mechanism for PSK enhancement of cytokine action (Sakagami *et al.*, 1991a).

4.4. Antiviral Activity

PSK has been shown to inhibit cytopathic effects of the human immunodeficiency virus (HIV) that infects cells of the CD4 positive human T cell line (Tochikura *et al.*, 1987). PSK almost completely blocked the giant formation and HIV-specific antigen expression in both MT-4 cells and MOLT-4 cells at concentrations of 400 and 800 μg/ml, respectively. PSK also non-competitively inhibited reverse transcriptase of avian myeloblastosis virus *in vitro*. One mechanism of the PSK effect is attributed to the inhibition of binding of HIV to the cells (Hirose *et al.*, 1987). However, the anti-HIV activity of PSK (EC_{50}=300 μg/ml) is much lower than that of natural lignin (EC_{50}=6-100 μg/ml) (Sakagami *et al.*, 1992d; Manabe *et al.*, 1992), synthethic lignin (dehydrogenation polymers of phenylpropenoids) (EC_{50}=1.3-13 μg/ml)(Nakashima *et al.*, 1992a), monomeric, dimeric, trimeric, tetrameric hydrolyzable tannins (EC_{50}=2.0-7.0 μg/ml) (Nakashima *et al.*, 1992b) or sulfated polysaccharides (EC_{50}=1.8 μg/ml) (Koizumi *et al.*, 1993). All of these agents significantly inhibited HIV binding to the cells.

However, the antiviral activity of PSK against other viruses is relatively weak. PSK (10 and 100 μg/ml) did not inhibit the plaque formation of the herpes simplex virus (Fukuchi *et al.*, 1989), or the influenza virus (Harada *et al.*, 1991).

4.5. Radical Scavenging Effect

PSK potently inhibited the luciferin-dependent chemiluminescence generated by opsonized zymosan-stimulated human peripheral blood PMN. The continuous presence of PSK was necessary to express its inhibitory activity. PSK also scavenged the chemiluminescence generated by the xanthine-xanthine oxidase reaction, or by potassium superoxide solution. The inhibitory activity of PSK (EC_{50}=100 μg/ml) was one-order lower than that of tannins and lignins. It is suggested that O_2^- might be scavenged directly by PSK, or by other oxygen radicals produced by activation of the MPO-dependent oxidative process in PMN (Sakagami *et al.*, 1992a).

PSK also scavenged the chemiluminescence generated by the reaction of sodium hypochlorite (a product of MPO reaction) and luminol, although the inhibitory activity of PSK (EC_{50}=26 μg/ml) was much lower that that of tannin-related compounds, or ligninified materials (EC_{50}=0.058-3 μg/ml) (unpublished data).

On the other hand, Schizophyllan and N,N-dimethylaminoethylparamylon did not scavenge O_2^- (Sakagami *et al.*, 1992a) or hypochlorite (unpublished data).

4.6. Inhibition of Poly(ADP-Ribose)Glycohydrolase Activity

Reversible poly-(ADP-ribosyl)ation of chromosomal proteins has been proposed to be important in the regulation of nuclear functions, such as DNA replication, repair and transcription, and thus affect cell growth and differentiation. Poly-(ADP-ribose)glycohydrolase, which splits the glycosidic (1"-2') linkages of poly-(ADP-ribose), is the main enzyme responsible for the degradation of poly-(ADP-ribose). No potent, specific inhibitors of this enzyme have been reported. Recently, it has been reported that dimeric hydrolyzable tannins suppressed this enzyme activity and the glucocorticoid-sensitive mouse mammary tumor virus (MMTV) mRNA in 34I mouse mammary tumor cells (Tsai *et al.*, 1992). Various polyphenols such as tannins (Tanuma *et al.*, 1989a) and lignins (Tanuma *et al.*, 1989b), were found to be potent inhibitors of poly-(ADP-ribose) glycohydrolase. Analysis of the kinetics revealed that inhibition by these polyphenols was competitive with respect to the substrate poly-(ADP-ribose). PSK also inhibited this enzyme activity via the same mechanism, but much less efficiently, whereas glucans were inactive (Tanuma *et al.*, unpublished data).

5. BIOLOGICAL ACTIVITY OF PSK SUBFRACTIONS

PSK was separated into the following four subfractions, of different molecular weights, by successive filtration through membrane filters: F1 (<50 kD), F2 (50-100 kD), F3 (100-200 kD) and F4 (>200 kD), with relative yields of 1 : 1 : 10 : 10 (Tsuchitani *et al.*, 1987). The highest molecular weight fraction (F4), among these fractions showed the greatest activity in the following items: (i) induction of antimicrobial activity in mice by pretreatment to reduce the lethal infectivity of *Escherichia coli* (Harada *et al.*, 1989), (ii) stimulation of the functional maturation (i.e., morphological change, increase of NBT-reducing activity) of mouse peritoneal macrophages (Kurakata *et al.*, 1991), (iii) stimulation of TNF- (IFN-γ)-induced differentiation of the human myelogenous leukemic cell lines, ML-1, HL-60, U-937, THP-1 (Sakagami *et al.*, 1989; Kim *et al.*, 1990), and (iv) iodination of human peripheral blood PMN (Sakagami *et al.*, 1990b).

Material which stimulated PMN iodination was precipitated between pH4.0 and 4.5 (Sakagami *et al.*, 1990b). Further purification and structural analysis of the active subfractions remain to be achieved.

6. CONCLUSIONS

PSK, unlike most of other glucans, displayed various unique biological activity: (i) stimulation of functional maturation of macrophages, (ii) stimulation of PMN iodination, (iii) inhibition of the cytopathic effect of HIV infection, (vi) inhibition of enzyme activity, and (v) ability to scavenge active oxgen (O_2^-, hypochlorite). Lignified materials (Sakagami *et al.*, 1991b), but not most other glucans tested, also displayed these properties. This suggests the presence of some unknown constituent(s), possibly lignin, in the PSK molecule. However, at present, the possibility that some slight difference in structural configuration, including sugar linkage, might confer unique biological activity on PSK cannot be eliminated.

It was suggested that some immunopotentiating activity displayed by PSK might be generated by enhancement of cytokine action, or by stimulation of cytokine production. We demonstrated that PSK, together with bacterial preparations such as OK-432 or *Lactobacillus casei*, stimulated some endogenous cytotoxic factor(s) or TNF. TNF, originally isolated as a macrophage-derived tumoricidal cytokine, has a broad spectrum of biological activity (Old, 1987). However, direct injection of this cytokine into host animals has occasionally induced shock and tissue injury (Tracy *et al.*, 1986). In contrast, administration of various biological BRMs, combined with appropriate eliciting agents of bacterial origin, has been reported to induce endogenous TNF in an amount sufficient to suppress tumor growth, without producing side effects. There is a broad parallel between the capacity of TNF production and the reduction of tumor weight (Haranaka *et al.*, 1988). Therefore, the ability of PSK, in combination with appropriate eliciting agents, to induce endogenous TNF (Sakagami & Takeda, 1992c) suggests the efficacy of PSK for clinical application to cancer patients.

PSK and lignins stimulated the iodination of MPO-positive cells. This suggests that these iodination stimulators might activate the MPO-halogen-H_2O_2 antibacterial system. Thus, it is possible that the antimicrobial activity of these substances might be augmented especially in MPO-positive cells. However, lignins almost equally inhibited acute infection of HIV in both HL-60 cells (which have higher MPO concentrations) and U-937 cells (which have much lower MPO concentrations) (Kunisada *et al.*, 1992). This indicates that the anti-HIV activity of these substances does not depend on the content of MPO in the cells. PSK may interfere with the early stages of HIV infection by modifying the viral receptor (Tochikura *et al.*, 1987), or it may directly inactivate the virus. The possibility that PSK might inhibit reverse transcriptase activity, inhibit viral expression from host DNA, as well as activate host immune functions remains to be investigated. Thus, PSK may be useful in preventing the risk of HIV infection, and in the treatment of AIDS.

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