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Anti-inflammatory properties of GLPss58, a sulfated polysaccharide from *Ganoderma lucidum*

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ABSTRACT

Sulfated polysaccharides exhibit various biological properties, including anti-coagulant, anti-oxidant, anti-viral, anti-cancer, anti-inflammatory and immune regulatory activities. In the present study, the anti-inflammatory properties of GLPss58, a sulfated polysaccharide from *Ganoderma lucidum* formed by chemical sulfation, were investigated. We found that GLPss58 inhibited L-selectin/sTyr-sLeX binding significantly, blocked the binding of anti-L-selectin antibodies to L-selectin on the surface of human peripheral blood lymphocytes, and inhibited the secondary lymphoid tissue chemokine-induced chemotactic invasion of HPBLs. *In vivo* studies in mice showed that lymphocyte homing from peripheral blood to spleen and lymph nodes was significantly inhibited by GLPss58. Furthermore, GLPss58 also inhibited the activation of complement systems and blocked the binding of TNF-α and IFN-γ to their antibodies. These results indicate that GLPss58 is able to inhibit not only the L-selectin-mediated inflammation, but also the complement system- and cytokines mediated-inflammation. Our results suggest that GLPss58 is a favorable potential anti-inflammatory agent.

1. Introduction

Ganoderma lucidum (*G. lucidum*) also known as "Ling Zhi" in China is a Lamellaless basidiomycete of the Polyporaceae family (Leyss. ex Fr. Karst). This medical fungus has been widely used for the treatment of various diseases in Chinese traditional medicine for more than 4000 years [1]. This mushroom has been suggested not only to prevent and treat diseases but also to be beneficial for general health and to prolong life [2].

G. lucidum has been reported to have a range of therapeutic properties including immunostimulatory, antioxidant, anti-inflammatory and anti-tumor effects [3]. Polysaccharides have been identified as one of the bioactive components in *G. lucidum* which have mostly been associated with immunomodulatory effects. GLIS, a bioactive proteoglycan from *G. lucidum* has been reported to stimulate the proliferation and activation of mouse B lymphocytes and to enhance the activity of mouse macrophages [4,5]. Polysaccharides isolated from the spores of *G. lucidum* have been demonstrated to have strong immunomodulatory activity especially on lymphocyte proliferation [6]. Another polysaccharide from the fresh fruit bodies of *G. lucidum* was demonstrated to have anti-tumor effects through stimulation of the activity of human monocytes macrophages and T-lymphocytes [7]. A study on mice showed that β-1,3/1,6-glucan from *G. lucidum* induced serum IgA and IgG production, enhanced poly-Ig receptor expression in the small intestine and increased IL-2 production by NK cells thereby reducing inflammation induced by a high-cholesterol diet [8].

It has been also reported that the *G. lucidum* extracts have anti-inflammatory effects *in vitro* and *in vivo*. The LZ-8 protein of *G. lucidum* induced expansion of Foxp3+ regulatory T (Treg) cells and alleviated acute intestinal inflammation in mice [9]. GLBR, an extract of *G. lucidum* grown on brown rice suppressed the

Abbreviations: BMMs, Bone marrow-derived macrophages; MSLs, mouse spleen lymphocytes; mAb, monoclonal antibody; SLC, secondary lymphoid tissue chemokine; SPR, surface plasmon resonance; HPBL, shuman peripheral blood lymphocytes; LN, lymph node.

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production of nitric oxide (NO) and prostaglandin E2 (PGE2) in lipopolysaccharide (LPS)-stimulated macrophages and decreased the expression of COX-2, TNF-α, IL-1β and IL-6 via inhibition of MAPK and NF-kB activation in a mouse model [10].

Many studies have reported that sulfated polysaccharides exhibit various biological properties including anti-coagulant, anti-oxidant, anti-viral, anti-cancer, anti-inflammatory and immune regulatory activities [11–15]. These include fucoidan, a sulfated polysaccharide with significant anti-inflammatory activity [16]. Dendritic polyglycerol sulfate (dOGS), a fully synthetic heparin analog, showed potent anti-inflammatory effects both *in vitro* and *in vivo* [17].

Previously we have reported that GLP20, a purified polysaccharide from fruiting bodies of *G. lucidum,* had an immunostimulatory effect on RAW264.7 mouse macrophage cells *in vitro* and determined that GLP20 has a structure comprised of a β-(1–3)-linked D-glucan with a β-(1–6)-D-glucopyranosyl side-branching unit on every third residue. In aqueous solution it has a rigid chain conformation with a triple-helix structure [18]. In the present study, the anti-inflammatory effects of GLPss58, a sulfated form of GLP20, were investigated.

2. Materials and methods

2.1. Preparation of GLPss58

GLP20, a β-D-glucan was purified from the fruit bodies of *G. lucidum* by extraction with boiled water and the following ethanol precipitation according to the previously reported method [18]. GLP20 was chemically modified to obtain its sulfated derivative (GLPss58) using the chlorosulfonic acid/pyridine method as reported [19]. The sulfation agent CSA/Pyr was prepared by dropping $HClSO_3$ one by one into anhydrous pyridine under agitating in a three-necked flask and cooling conditions in ice water bath. In brief GLP20 (0.50 g) was suspended in 50 ml of anhydrous pyridine at room temperature with vortex stirring and the sulfating reagent was added drop wise. The mixture was stirred for 4 h at 60° C and cooled down to room temperature and neutralized with 5 mol/L NaOH solution. Then the mixture was dialyzed against distilled water for 48 h to remove pyridine, salt and some potential degradation products. The dialysate was concentrated and precipitated with 80% ethanol. The precipitate was dissolved in distilled water and freeze-dried to obtain the final sulfated derivative designated as GLPss58. The Sulfur content (S%) in the sulfated sample was determined using barium sulfate turbidimetry method and the degree of substitution (DS) which refers to the average number of sulfate residues on each monosaccharide residue was calculated to be 0.58 according to the following equation,

$$DS = \frac{1.62 \times S\%}{32 - 1.02 \times S\%}$$

2.2. Animals

C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Animals were treated according to the German law on the protection of animals and approval (T0169/11) was obtained from the State Animal Welfare Committee. Male mice of 8–13 weeks of age were used for preparation of bone marrow-derived macrophages (BMMs) and mouse spleen lymphocytes (MSLs) according to the procedure by Zhang et al. [5].

2.3. Human blood

Human blood was obtained from healthy volunteers from the blood bank of Charité-Universitätsmedizin Berlin. Human blood collection was performed according to German Ethics Laws and approval (EA4/106/13) was obtained from the Ethics Committee of the Charité-Universitätsmedizin Berlin.

2.4. Measurements of L-selectin – sLex-sTyr binding in vitro by SPR

Surface plasmon resonance (SPR) experiments were carried out on a BIAcore X instrument (GE Healthcare) at 25 °C. The competitive selectin ligand binding assay has been described in detail previously [20]. L-selectin-IgG chimera (R&D Systems GmbHGermany) was immobilized on Protein-A-coated gold nanoparticles (15 nm diameters Biotrend Chemikalien GmbH Germany). Biotinylated synthetic L-selectin ligand SialeX-PAA-sTyr, in which the molar ratio of SialeX is 20% while that of sTyr is 5%, was prepared at concentration at 4.2 μg/ml in HBS-EP (Biacore AB consisting of 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% Surfactant P20) and immobilized on a sensor chip SA (Biacore AB). For reference purposes same concentration of PAA: poly [*N*-(2-hydroxyethyl) acrylamide] (Lectinity Holdings Inc. Russia) and N-acetyl-lactosamine-PAA (Lectinity Holdings Inc. Russia) were immobilized on a first and second lanes of the same chip respectively. The L-selectin coated nanoparticles were dispersed in 20 mM HEPES buffer (pH 7.4 with 150 mM NaCl and 1 mM $CaCl_2$) with final working concentration of 2 nM and injected over the reference lanes and the $SiaLe^x$-PAA-sTyr lanes at a flow rate of 20 μl/min. Reference lane data were subtracted from $SiaLe^x$-PAA-sTyr lane data, which was set to 100% and served as a control. To evaluate the influence of GLPss58 on L-selectin–sLex-sTyr binding, the L-selectin coated nanoparticles were incubated at first with GLPss58 for 30 min and then injected to pass over the sensor chip surface. Reduction of the binding signal with respect to the GLPss58 concentration was recorded and calculated as% binding of the control. The inhibitor concentration that caused 50% reduction of binding was referred as the IC_{50} value.

2.5. Analysis of binding of GLPss58 to L-selectin on the HPBL surface

Human peripheral blood lymphocytes (HPBLs) were isolated from fresh peripheral blood by Ficoll density gradient centrifugation according to the protocol of manufactory (Ficoll-Paque PLUSGE Healthcare Uppsala Sweden). Each 1×10^5 HPBLs were incubated with 10 μl of GLPss58 in PBS at indicated concentrations or the same volume of PBS as negative control for 30 min at room temperature in 90 μl RPMI 1640 medium containing 10% FCS (fetal calf serum). After washing two times with PBS, cells were labeled with FITC-conjugated anti-CD62L monoclonal antibody (mAb HuDERG-55, BD Biosciences, Germany) for 30 min on ice in 100 μl PBS. The cells were washed twice to remove unbound antibodiesand the number and the FITC-intensity of antibody bound HPBLs were determined by flow cytometry (FACS Calibur, BD Biosciences).

2.6. Analysis of SLC-induced chemotaxis of HPBLs

Secondary lymphoid tissue chemokine (SLC) (ImmunoTools, Germany) was suspended in RPMI 1640 medium containing 10% FCS at a concentration of 10 ng/ml. Chemokine (200 μl) was loaded into the lower wells of a chemotaxis assay chamber (CytoSelect™ 24-Well, 5 μM pore, Cell Biolabs). HPBLs (1×10^6 cells) were treated with PBS (as control) or with 50 μg/ml GLPss58 for 30 min, then washed twice and finally resuspended in 100 μl medium. The cells were then added to the upper wells of the chemotaxis assay chamber. The upper wells were separated from the lower wells by a polycarbonate membrane with 5 μm pores. The chemotaxis chamber was incubated at 37 °C for 2 h in a humidified incubator with 5%