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ORIGINAL ARTICLE

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Identification of the protein components of protein-bound polysaccharide (PSK) that interact with NKL cells

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Abstract We identified the protein components of a protein-bound polysaccharide (PSK) that are responsible for the biological function of this immunomodulator in its interaction with NKL cells, an NK-derived cell line previously known to be activated by this extract, obtained from the basidiomycete *Coriolus versiocolor*. In addition, we show that PSK protein interacts with NKL cells through a different receptor from that used by IL-2. This was deduced from the different molecular weights of the PSK/NKL and IL-2/NKL receptor complexes. We show that PSK is composed of a highly glycosylated 12-kDa protein. Protein-bound polysaccharide interacts in vitro with an NKL receptor of approximately 48 kDa, whereas IL-2 shows a similar interaction with NKL receptor proteins of approximately 64 and 75 kDa. Our results may explain why PSK and IL-2 use completely different intracellular routes for their biological activities in NKL cells—i.e., regulating different PKC isozymes, mitogen-activated protein kinases, and nuclear transcription factors.

Keywords IL-2 · Immunomodulator · NK activation · Protein-bound polysaccharide · PSK

Introduction

Protein-bound polysaccharide (PSK) obtained from basidiomycetes and also known as Krestin, has been used as a chemoimmunotherapy agent in the treatment of cancer in Asia for over 30 years [15, 17, 19, 21]. This polysaccharide is derived from the CM.101 strain of the fungus *Coriolus versicolor*, and has shown anticancer activity in vitro, in experimental models, and in human clinical trials [5, 12]. Several randomized clinical trials have demonstrated that PSK has great potential as an adjuvant cancer therapy agent, with positive results seen in the adjuvant treatment of gastric, esophageal, colorectal, breast, and lung cancers [11, 15, 16]. These studies have suggested the efficacy of PSK as an immunomodulator or biological response modifier. The precise molecular mechanism responsible for its biological activity has not yet been elucidated.

Protein-bound polysaccharide has also been shown to cause differentiation of cells in vitro, and this effect has been attributed to the induction of differentiation cytokines [9, 18, 20]. Some studies indicate that PSK acts as an antioxidant, which may allow it to play a role as a normal tissue chemoprotector and radioprotector when used in combination with adjuvant or definitive chemotherapy or radiotherapy to treat cancer [3, 14, 22]. Interestingly, several studies have also shown that PSK may actually inhibit carcinogenesis by inhibiting the action of various carcinogens in vulnerable cell lines [5]. In addition, an antimetastatic action of PSK has been demonstrated, and has been attributed to its potential to inhibit metalloproteinases and other enzymes involved in metastatic [5] and immunomodulator activity [1, 2].

Some studies indicate that PSK may act to increase leukocyte activation and response through the upregulation of key cytokines. Indeed, natural killer and lymphocyte-activated killer (LAK) cell activation has been demonstrated in vivo and in vitro [10, 23]. In this context, we have also shown that PSK is capable of inhibiting metastatic colonization in vivo in some experimental fibrosarcomas, and that this effect is mediated by the activation of NK cells [1]. Moreover, the NK cell line NKL, derived from a large granular lymphocyte leukemia [24], is activated in vitro by PSK [23]. This activation may replace IL-2 in the induction of proliferation and cytotoxicity in NKL cells. We have demonstrated that the signal transduction pathway involved in the responses to IL-2 or PSK is different. In protein kinase C (PKC) isozymes, IL-2 increased the expression of PKCα, whereas

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PSK decreased the expression of this isozyme. In studies of mitogen-activated protein kinase expression we found that IL-2 decreased the expression of ERK2, whereas PSK did not, and both agents increased the expression of ERK3 [8]. PSK also enhanced CRE-binding activity, whereas IL-2 increased SP-1 and modified GAS/ISRE, IRF-1, and STAT5 [7]. These results suggest that PSK and IL-2 bind to a different receptor in NKL cells.

These biological activities are no doubt mediated by one or more PSK ligands that interact with a PSK receptor present on NKL cells. The present study was designed to identify the protein component(s) of PSK and their interaction with NKL cell receptors. The interaction between NKL and IL-2 was used as an external positive control.

Materials and methods

Protein-bound polysaccharide K

Protein-bound polysaccharide K was kindly provided by Kureha Chemical Industry (Tokyo, Japan). It is prepared by extracting cultured mycelia of *C. versicolor* with hot water. The precipitate is separated from the clear supernatant with saturated ammonium sulfate, then desalted and dried [25]. Protein-bound polysaccharide K was dissolved in RPMI medium or water and heated at 50°C for 20–30 min until a clear solution appeared. The PSK preparation was filter-sterilized and diluted in culture medium or water to the desired concentration. Protein-bound polysaccharide K was titrated previously in NKL cells [23] and the working dilution we used was 100 μg/ml.

Culture of NK cells, cytokines, and reagents

Cell line NKL [24] was established from peripheral blood lymphocytes of a patient with LGL leukemia by Dr J. Ritz, and was kindly provided by Dr M. Lopet-Botet (Hospital de la Princesa, Madrid, Spain). NKL cells were maintained in culture with RPMI 1640 and 10% heat-inactivated human AB serum (Sigma Chemical, St Louis, MO, USA). Recombinant human IL-2 was purchased from Hoffmann–la Roche (Nutley, NJ, USA) (purity >97%, specific activity, 2×10^6 U/mg). Unit definition: one unit is the amount required to induce half-maximal incorporation of [^{3}H]thymidine into IL-2–dependent cytolytic T-cell lymphocytes. For all assays the NKL cell line was cultured in medium supplemented for 96 h with either human recombinant IL-2 (1,000 U/ml) or PSK (100 μg/ml).

Protein extraction

Protein extracts were obtained from NKL cells incubated for 96 h with IL-2 or PSK. Briefly, 10^6 cells were collected, washed twice in phosphate-buffered saline, and lysed in 1 ml of lysis buffer (1% [vol/vol] Triton X-100, 50 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM EDTA [pH 8], 1 mM Na_3VO_4, 50 mM NaF, 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin). After incubation during 10 min at room temperature with constant rotation, lysates were cleared of debris by centrifugation at 10,000 *g* for 30 min at 4°C. Protein concentration was determined with the Bradford protein assay (Bio-Rad, Madrid, Spain).

Neuraminidase digestion

One hundred micrograms of PSK was digested with 4 μl neuraminidase (Sigma Chemical, St Louis, MO, USA) and incubated 3 h at 37°C. Digested PSK was labeled with [^{35}S]methionine.

Protein labeling protocol

IL-2 or PSK were labeled in vitro with [^{35}S]methionine by lyophilization according to the method published by Browder et al. [4]. Proteins to be labeled were dissolved in double-distilled water, added to [^{35}S]methionine (Amersham, cat. no. SJ204; specific activity >800 Ci/mmol) in 1.5 ml microcentrifuge tubes, and lyophilized. Typical labeling protocols used 60–90 μCi to [^{35}S]methionine, 1–20 μg protein, and double-distilled water in a total volume before lyophilization of 20 μl. After lyophilization, the proteins were redissolved in 10 μl 50 mM HEPES-NaOH buffer, pH 7.4. To finish the reaction and to separate the unreacted label from the radiolabeled protein, the reaction mixture was passed though a Sephadex G-25 column. In some experiments, the labeled IL-2 or PSK was incubated in vitro with total protein extracts from NKL cells induced with IL-2 or PSK. Incubation was at 37°C, with agitation during 2 h.

SDS-polyacrylamide gel electrophoresis and fluorography

Each sample with labeled proteins was added to an equal volume of Laemmli sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, and 0.01% blue bromophenol. In experiments performed under reducing conditions, 5% 2-mercaptoethanol was added to this buffer. After the samples were boiled for 3 min, equal volumes of solutions of labeled proteins were loaded into wells of 8%, 12%, and 15% polyacrylamide gels with 5% stacking gels, and electrophoresed using the buffer system of Laemmli [13]. After electrophoresis, the gels were fixed in 10% acetic acid and 45% methanol in distilled water, and then soaked in Amplify (Amersham) during 15 min. The gels were dried and bands were detected after exposing the gels to Kodak XAR film at −80°C (Eastman Kodak Company, Rochester, NY, USA).

Results

Identification of the protein components of PSK by SDS-PAGE

Protein-bound polysaccharide has a protein component that is not stained with Coomassie Brilliant Blue after SDS-PAGE [6], or with silver staining (data not shown). To identify the molecular weight of the protein component of PSK by SDS-PAGE, we labeled the proteins by lyophilization together with [^{35}S]methionine in double-distilled water. The proteins were dissolved in buffer, passed through a Sephadex G-25 column, and subjected to SDS-PAGE and fluorography.

We used a similar labeling method in vitro with IL-2. Three micrograms of IL-2 was labeled with 60 μCi [^{35}S]methionine. After lyophilization the sample was redissolved in 10 µl HEPES-NaOH buffer, pH 7.4. This sample was added to an equal volume of ×2 SDS sample buffer with 2-mercaptoethanol, boiled for 3 min, loaded on 10% polyacrylamide gel and electrophoresed. Figure 1a shows a band of 15 kDa detected by this method.

Protein-bound polysaccharide was labeled with 2 and 5 µg, ×2 SDS sample buffer with or without 2-mercaptoethanol. Fluorography showed two bands of very high molecular weight (Fig. 1b). To determine whether this high molecular weight band was highly glycosylated or strongly bound to polysaccharides, PSK was digested with neuraminidase before labeling. The band disappeared completely and a new band of about 12 kDa appeared (Fig. 1c). These results indicate that PSK is composed of a single protein, and that the two previously characterized bands corresponded to two different patterns of glycosylation.

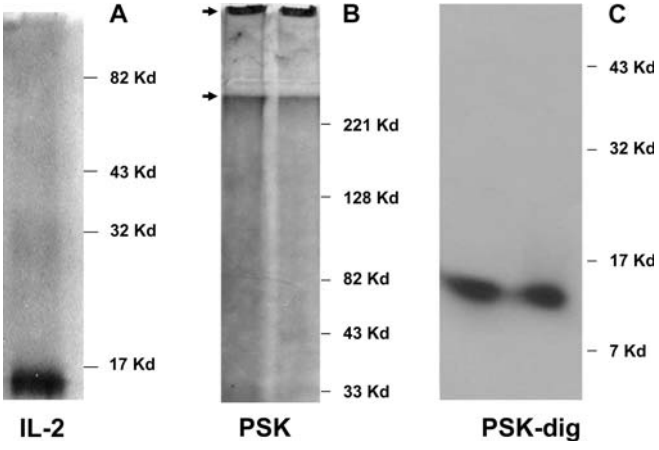

Fig. 1 Molecular weight of protein component of IL-2 and PSK. **a** SDS-PAGE of labeled IL-2 showed a 15-kDa protein. **b** SDS-PAGE of labeled PSK showed two high molecular weight bands. **c** A single new band of about 12 kDa appeared after digestion of PSK with neuraminidase. The experiments were performed at least three times obtaining the same results, and representative data are shown

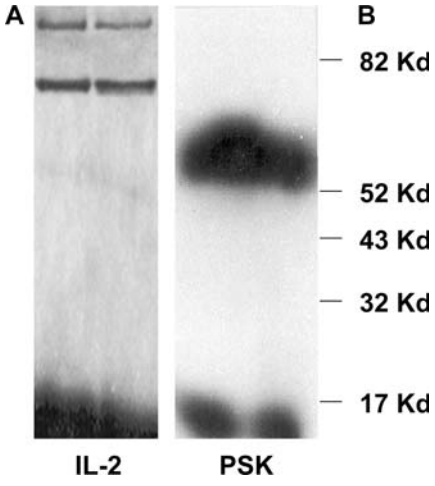

Fig. 2 Interaction of IL-2 and PSK with receptor proteins derived from NKL cells. **a** IL-2 interacts with two proteins of about 65 and 75 kDa. **b** PSK interacts with a single protein of about 48 kDa. The experiments were performed at least three times obtaining the same results, and representative data are shown

Interaction of IL-2 and PSK with receptor proteins derived from NKL cells

To investigate whether the protein component of PSK and IL-2 binds to the same receptor on NKL cells, we tested the interaction or binding of the protein component of PSK and IL-2 to proteins derived from NKL cells. Protein extracts were obtained from NKL cells and treated with PSK or IL-2.

Labeled IL-2 (3 and 6 µg) was incubated for 2 h in vitro with proteins derived from NKL cells. After SDS-PAGE without 2-mercaptoethanol and fluorography, the results showed that in addition to the band corresponding to IL-2, new bands appeared with an approximate molecular weight of 80 and 90 kDa (Fig. 2). These two new bands did not disappear when the incubation was performed in presence of PSK, indicating the absence of competition. These results indicated that IL-2 interacts with two proteins of approximately 65 and 75 kDa, this interaction being specific for IL-2. When similar amounts of labeled PSK were incubated with proteins derived from NKL cells, in addition to the 12-kDa band, a new band of about 60 kDa appeared (Fig. 2). The presence of IL-2 during the assay did not compete with PSK, and the 60 kDa was again detected. These results clearly indicated that PSK interacts with a receptor protein of about 48 kDa derived from NKL cells, and that this interaction involves a different receptor from the one used by IL-2.

Discussion

We have identified the protein component of PSK by labeling PSK in vitro with [^{35}S]methionine via a lyophilization procedure. It was previously known that

Coomassie Brilliant Blue [6] and silver did not stain PSK. We have found that PSK is composed of two bands of very high molecular weight. After digestion with neuraminidase, these bands are reduced to a single band of about 12 kDa (Fig. 1). These results indicate that PSK is probably composed of a single 12-kDa protein, and that this protein is highly glycosylated (Fig. 1).

After in vitro incubation of PSK with protein extracts derived from NKL cells, a new band of about 60 kDa appeared, indicating that its receptor on NKL cells has a molecular weight of approximately 48 kDa (Fig. 2). This receptor is different from the IL-2 receptor, which has a molecular weight of approximately 64 and 75 kDa (Fig. 2). These molecular weights correspond to the γ and β subunits of the IL-2 receptor, are known to be expressed in NK cells [26].

We previously reported that PSK and IL-2 induce different signaling pathways on NKL cells [7, 8]. In light of the present findings, this suggests that PSK and IL-2 bind different receptors on NKL cells. In fact, our results confirm that IL-2 does not interfere with the interaction between PSK and its NKL receptor, nor does PSK interfere with IL-2 and its receptor in NKL cells. In this context, it was recently shown in NOR-P1 human pancreatic cancer cells that PSK increases docetaxel-induced apoptosis by inhibiting the NF-κB transcription factor [27]. It is possible that PSK may act on different molecular targets depending on the cellular type—i.e., enhancing activity on NKL cells or inducing apoptosis of pancreatic cancer cells.

Protein-bound polysaccharide has been reported to suppress metastases in artificial models such as mouse fibrosarcoma [1], rat hepatoma AH60C, and mouse cancer colon 26 [11]. The metastatic process is a highly complex process involving the activation and inactivation of multiple genes that are associated with cell motility and innate and adaptive immune responses. Our previous findings, as well as this report, strongly suggest that PSK exerts its biological activity as an immunomodulator by enhancing the NK cell immune response [1, 7, 8]. Further characterization of the protein component of PSK and its interactions with NK cells and T lymphocytes will help us to understand the biological relevance of PSK in cancer immunotherapy.

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