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ORIGINAL ARTICLE

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Different regulation of PKC isoenzymes and MAPK by PSK and IL-2 in the proliferative and cytotoxic activities of the NKL human natural killer cell line

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Abstract The activation of natural killer (NK) cells and induction of cytotoxicity are complex processes whose molecular mechanisms have not been clearly elucidated. Stimulation of the NKL human NK cell line with interleukin-2 (IL-2) or protein-bound polysaccharide K (PSK) leads to sustained growth and cytolytic activity in comparison to unstimulated NKL cells. Our previous results shown that IL-2 and PSK regulate different nuclear transcription factors in NKL cells, and that the signal transduction pathway used by these inducers is different. To determine the molecular basis for the different action of IL-2 and PSK, we investigated the upstream effects generated in human NKL cells by IL-2 and PSK on protein kinase C (PKC) isoenzymes and mitogen-activated protein kinases (MAPK). Here we report the profile of unstimulated NKL cells as: PKCβ > PKCα > PKCδ = PKCϵ. The PKCη form was not expressed. The effects of PSK and IL-2 on these isoenzymes were different. IL-2 increased the expression of PKCα, PKCδ and PKCϵ, whereas PSK decreased the expression of PKCα, and also increased PKCδ and PKCϵ to higher levels than did IL-2. In MAPK expression we found that unstimulated NKL cells have the following profile: ERK2 > ERK6 > p38γ > p38β > ERK1. ERK3, ERK3 rel, ERK5/ERK4 and p38δ were not expressed. IL-2 decreased the expression of ERK2, whereas PSK did not, and both agents increased the expression of ERK3. These results shown that PSK and IL-2 produce different variations in PKC isoenzymes and MAPK in NKL cells.

Keywords Cancer immunotherapy · Interleukin-2 · MAPK · NK cell · PKC isoenzyme · PSK

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Introduction

Polysaccharide K (PSK), a protein-bound polysaccharide, is widely used in Japan as a biological response modifier for cancer patients [15, 16, 18]. This polysaccharide is obtained from the basidiomycete fungus *Coriolus versicolor*. Its antitumor effect is believed to derive from its immunomodulatory activity on the tumor-bearing host [17, 34]. However, the exact mechanisms of its immunomodulating effect are not well known. We have previously shown that PSK is able to inhibit metastatic colonization in a mouse fibrosarcoma model, and that this inhibition is due to the activation of natural killer (NK) cells in vivo [1, 2].

To further analyze the mechanisms responsible for this NK activation we studied the effect of PSK in the human NK cell line NKL, derived from a patient with large granular lymphocyte leukemia (LGL), which showed natural cytotoxic activity [22]. PSK can substitute for interleukin-2 (IL-2) in the induction of proliferative and cytotoxic activities in the NKL cells [19]. We have also previously shown that the signal transduction pathway induced by these agents is different, as they lead to different variations in the binding activities of some transcriptional factors. Only treatment with PSK increases cyclic AMP response element (CRE) binding activity, whereas only treatment with IL-2 increases SP-1 and modifies GAS/ISRE, IRF-1 and STAT5 binding activities [6]. In addition, it has been shown that PSK can activate human NK cells independently of the IL-2/IL-2R system [11].

The protein kinase C (PKC) isoenzymes and mitogen-activated protein kinases (MAPK) have been widely reported to be involved in the proliferation, activation and natural cytotoxicity of NK cells [4, 24]. The PKC family of homologous serine/threonine protein kinases is involved in a number of processes such as growth, differentiation and cytokine secretion [10, 13, 14, 25, 29]. Different tissues seem to have their own characteristic patterns of PKC isozyme expression. This

had led to the hypothesis that each isozyme has a different biological function. Activation of MAPK family members has been implicated in various immune functions [4, 9, 21]. Extracellular signal-regulatory kinases (ERK) and p38 kinase activity are necessary for proper T cell development [23, 26] and for IL-2 gene transcription in response to T cell receptor (TCR) stimulation [33]. In NK cells, the ERK enzyme has been implicated in NK killing and lymphokine-activated killing (LAK) by IL-2 [30, 32, 35].

To determine the molecular mechanisms involved in proliferation and NK cytotoxicity induced by PSK in NKL cells, and to compare these mechanisms with those used by IL-2, we monitored the levels of several PKC isozymes and MAPK in unstimulated NKL cells and in NKL cells treated with PSK or IL-2. In this study, we show that PSK and IL-2 lead to different variations in some of these enzymes in NKL cells. IL-2 increases the expression of PKCα, whereas PSK decreases its expression and increases PKCϵ expression more strongly than does IL-2. In MAPK only IL-2 decreases ERK2, whereas PSK decreases p38γ to lower levels than those seen after incubation with IL-2.

Materials and methods

Protein-bound PSK

Protein-bound PSK was kindly provided by Kureka Chemical Industry (Tokyo, Japan). It is prepared by extracting cultured mycelia of *Coriolus versicolor* with hot water. The precipitate is separated from the clear supernatant with saturated ammonium sulfate, then desalted and dried [31]. Protein-bound PSK was dissolved in RPMI medium and heated at 50°C for 20–30 min until a clear solution was obtained. The PSK preparation was filter-sterilized and diluted in culture medium to the desired concentration. Protein-bound PSK was previously titrated in NKL cells [19], and the working dilution used was 100 µg/ml.

Culture of NK cells, cytokines and reagents

Cell line NKL [22] was established from peripheral blood lymphocytes (PBL) of a patient with LGL leukemia by J. Ritz, and was kindly provided by M. Lopet-Botet (Hospital de la Princesa, Madrid, Spain). NKL cells were maintained in culture with RPMI 1640 medium and 10% heat-inactivated human AB serum (Sigma Chemical, St. Louis, Mo.). Recombinant human IL-2 was purchased from Hoffmann-la Roche (Nutley, N.J.; purity > 97%, specific activity, 2×10^6 U/mg). Unit definition was as follows: one unit was 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 alone (unstimulated cells) or supplemented for 96 h with either human recombinant IL-2 (1,000 U/ml) or PSK (100 µg/ml).

In vitro NK cytotoxicity assays

Cytotoxicity tests in treated and untreated NKL cells were performed with a standard 2–3-h ^{51}Cr-release assay against Daudi tumor cells. Spontaneous release of radioactivity, determined by incubating the target cells in 100 µl of medium, was always less than 20% of maximum release; the variation between replicates was always less than 15%. The results were expressed as a percentage of specific lysis.

Western blot assays

Protein extracts were obtained from unstimulated NKL cells not treated during a 96-h period, or the NKL cells incubated for 1 h or 96 h with IL-2 or PSK. Briefly, 10×10^6 cells were collected, washed twice in phosphate-buffered saline (PBS), 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]. Lysates were cleared of debris by centrifugation at 14,000 *g* for 15 min, and the protein concentration was determined with the Bradford protein assay (Bio-Rad, Madrid, Spain). Equal amounts of protein extract (20 µg protein/lane) were electrophoresed on 10% SDS-PAGE. Proteins were electrophoretically transferred onto nitrocellulose membranes (Bio-Rad) using the Semi-Phor semidry transfer system (Hoefer, San Francisco, Calif.). After nonspecific reactivity was blocked with 5% nonfat dried milk in TBS-T (20 mm Tris-HCl, pH 7.5; 150 mM NaCl, and 0.01% Tween-20), filters were incubated for 1 h at room temperature with the following specific monoclonal antibodies (mAb) diluted in TBS-T: anti-PKCα at a 1:1,500 dilution, anti-PKCβ at a 1:500 dilution, anti-PKCδ at a 1:1,000 dilution, anti-PKCϵ at a 1:1,000 dilution, and anti-PKCη at a 1:500 dilution. All mAb were obtained from Becton Dickinson (San Jose, Calif.). Filters were then processed using a chemiluminescence kit (ECL; Amersham, Freiburg, Germany), and exposed to Hyperfilm ECL films (Amersham). Band intensity was scored by scanning densitometry (Kodak Digital Science ID image analysis software). The experiments were performed at least three times.

RNAse protection assays

mRNA was extracted from unstimulated NKL cells over 96 h, and from PSK- and IL-2- stimulated NKL cells over 1 h or 96 h, using the mRNA isolation kit (Roche, Indianapolis, Ind.). The expression of different MAPK was detected using the hMAPK Multi-Probe Template Set (Pharmingen, San Diego, Calif.) with the Riboquant Ribonuclease Protection Assay System (Pharmingen). The hMAPK template set contained DNA templates for ERK1, ERK2, ERK3, ERK3 rel, ERK5/ERK4, ERK6, p38γ, p38β, p38δ, GAPDH and L32. The constitutively expressed genes L32 and GAPDH were used as internal standards. Briefly, template DNA was used to generate ^{32}P-labeled riboprobes of defined length with T7 RNA polymerase in the presence of 100 µCi [α^{32}P]UTP (Pharmacia-Amersham, Amersham, U.K.) for 60 min at 37°C. The reaction was terminated by the addition of RNAse-free DNAse. Labelled RNA was extracted by phenol-chloroform followed by ethanol precipitation. The probes (0.5 to 1.0×10^6 cpm) were hybridized overnight at 56°C with 500 ng mRNA of unstimulated or stimulated NKL cells. After hybridization, the mixture was treated with RNAse A + T1 mix (Pharmingen) to digest the unhybridized single-stranded RNA. The protected fragments were then purified by phenol/chloroform extraction and ethanol precipitation. The samples were resuspended in 5 µl loading dye and resolved on a 6% acrylamide/8 M urea gel. The gels were dried, and protected bands representing different MAPK were detected after exposure of the gels to Kodak XAR film (Eastman Kodak, Rochester, N.Y.). Band intensity was scored by scanning densitometry (Kodak Digital Science ID image analysis software). The experiments were performed at least three times.

Results

PKC isozyme expression

We analyzed the expression of the PKC isozymes PKCα, PKCβ, PKCδ, PKCϵ and PKCη. The involvement of these PKC isozymes in the proliferation and natural

cytotoxicity of NKL cells was investigated by comparing the expression in protein extracts prepared from NKL cells alone and from NKL cells treated with IL-2 or PSK. We have previously reported that NKL cells stimulated by PSK continue to proliferate and show cytotoxic activity [19]. Before preparation of protein extracts, we divided the samples into two aliquots and measured the viability and cytotoxic activity of unstimulated NKL cells and NKL cells treated with IL-2 or PSK over a 96-h period. Viability of NKL cells was determined by trypan blue staining, and cytotoxic activity was measured in a standard 3-h ^{51}Cr release assay against a Daudi target tumor cell line. There were no significant differences in viability, proliferation or cytotoxic activity between NKL cells treated with IL-2 or PSK (data not shown).

Expression was measured by western blot, using mAb against each of the PKC isoenzymes. Protein extracts were obtained from untreated NKL cells after 96 h of culture, or from NKL cells treated with IL-2 or PSK for 1 h or 96 h. The results obtained from protein extracts obtained 1 h or 96 h after induction were identical. Unstimulated NKL cells expressed PKCα, PKCβ, PKCδ and PKCϵ, and did not express PKCη (Fig. 1). The pattern of expression was: PKCβ > PKCα > PKCδ = PKCϵ (Fig. 1). The NKL natural killer cells treated with IL-2 showed induction of expression of PKCα, PKCδ and PKCϵ, and no variation in the expression of PKCβ (Fig. 1). In contrast, NKL cells treated with PSK showed induction of the expression of PKCϵ to higher levels than those found after IL-2 treatment, the same degree of induction of PKCδ expression, decreased expression of PKCα, and no variation in PKCβ expression (Fig. 1). Comparing the results obtained in IL-2-treated cells with PSK-treated cells, we found a higher expression of PKCα in IL-2-stimulated cells, and a higher expression of PKCϵ in NKL cells treated with PSK, whereas PKCβ and PKCδ showed the same expression in both cells (Fig. 1).

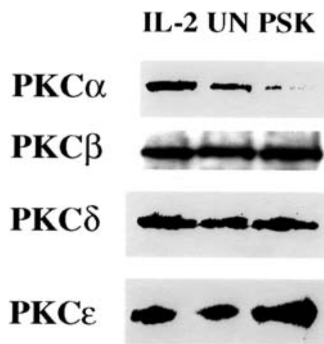

Fig. 1 Effects of IL-2 and PSK on PKC isoenzyme expression in NKL cells. Protein total extracts prepared from NKL cells grown without stimulation for 4 days (*lane UN*), IL-2-stimulated NKL cells (*lane IL-2*) or PSK-stimulated NKL cells (*lane PSK*) were separated by SDS-PAGE, and membranes were incubated with mAb for PKCα, PKCβ, PKCδ, PKCϵ and PKCη. No expression was found for PKCη. The experiments shown are representative of three independent assays

Expression of MAPK in NKL cells

The MAPK analyzed were ERK1, ERK2, ERK3, ERK3 rel, ERK5/ERK4, ERK6, p38γ, p38β, and p38δ. We analyzed the expression of mRNA by ribonuclease protection assay, using radiolabeled antisense RNA probes for each of these enzymes. mRNA was extracted from unstimulated NKL cells for 96 h, and the NKL cells were treated with IL-2 or PSK for 1 h and 96 h. The results obtained with both induction times were identical. Before preparation of mRNA, we divided the samples into two aliquots and measured viability and cytotoxic activity of unstimulated NKL cells and NKL cells treated with IL-2 or PSK for 96 h. Viability of NKL cells was determined by trypan blue staining, and cytotoxic activity was measured in a standard 3-h ^{51}Cr release assay against a Daudi target tumor cell line. There were no significant differences in viability, proliferation or cytotoxic activity between NKL cells treated with IL-2 or PSK (data not shown).

In untreated NKL cells, we found mRNA expression of ERK1, ERK2, ERK6, p38γ and p38β (Fig. 2). The profile of expression was ERK2 > ERK6 > p38γ > p38β > ERK1. When NKL cells were treated with IL-2, the expression of ERK3 was upregulated and the expression of ERK2 was dowregulated, together with that of ERK6, p38β and p38γ (Fig. 2). Treatment with

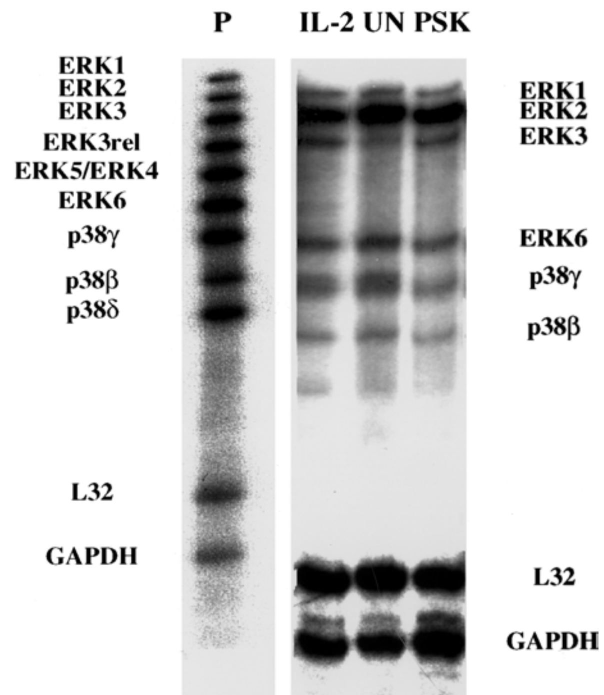

Fig. 2 Expression of MAPK in unstimulated and stimulated NKL cells. mRNA was extracted from unstimulated NKL cells (*lane UN*) and IL-2- and PSK-stimulated NKL cells (*lanes IL-2 and PSK*), and was incubated with labeled probes for different MAPK. L32 and GAPDH genes were used as controls. The results shown are from one of three independent experiments

Table 1 Summary of the expression of PKC isoenzymes and MAPK

	PKCα	PKCβ	PKCδ	PKCϵ	PKCη	
Unstimulated	+	+ + + +	+ +	+	–	
IL-2	+ + +	+ + + +	+ + +	+ +	–	
PSK	+/–	+ + + +	+ + +	+ + + +	–	
	ERK1	ERK2	ERK3	ERK6	p38β	p38γ
Unstimulated	+	+ + + +	–	+ + +	+ +	+ + +
IL-2	+	+ +	+ +	+ +	+	+ +
PSK	+	+ + + +	+ +	+ +	+	+

Results are graded as + + + + (100% positive expression), + + + (75% positive expression), + + (50% positive expression), + (25% positive expression), and – (negative expression)

PSK upregulated the expression of ERK3, downregulated ERK6 and p38β, and also downregulated p38γ to a lower level than that seen after treatment with IL-2 (Fig. 2). To verify the quality of mRNA and check that the amount was the same in each reaction, each mRNA was hybridized with L32 and GADPH probes (Fig. 2).

The main differences between PSK and IL-2 in MAPK regulation were that only IL-2 decreased the expression of ERK2, whereas PSK induced a greater downregulation of p38γ (Fig. 2).

Discussion

In the present study, we show that PSK decreases the expression of PKCα whereas IL-2 increases its expression. Both agents increase the expression of PKCδ and PKCϵ, but PSK increases PKCϵ more strongly (Fig. 1 and Table 1). Only treatment with IL-2 decreases ERK2 expression, while both agents increase ERK3 expression and decrease p38 (Fig. 2 and Table 1). When both stimuli, PSK and IL-2, were used together there was no synergistic effect, and the results obtained were identical to the combined results obtained when both stimuli were used independently (data not shown).

The specific PKC isoenzymes regulate different cellular functions in stimulated human lymphocytes: PKCα and PKCθ are involved in the expression of the IL-2 receptor [10, 27], whereas PKCβ, PKCδ and PKCϵ are involved in IL-2 synthesis and in the IL-2-mediated proliferation of T cells [8, 28]. In NK cells, the rapid activation of PKC enables them to mediate natural cytotoxicity towards tumor cells [24, 25], and PKC activation is essential for triggering lysis in IL-2-activated LAK cells [13]. In NKL cells, PKCα seems likely to be involved in the signal transduction pathway produced by IL-2. In contrast, PKCϵ might be involved in the transduction signal pathway produced by PSK. The PKCδ isoform might be involved in both pathways.

IL-2 stimulates ERK and p38 MAPK in various immune cell populations [3, 7, 12, 21], although the functional roles that these kinases play are still unclear. It has been shown that IL-2 can activate MAPK kinase (MKK)/ERK and p38 in lymphoid cell lines and primary T lymphocytes only after antigen stimulation [5, 12, 20]. However, in freshly isolated NK lymphocytes without any prior stimulation, IL-2 is able to activate the MKK/ERK pathway within a few minutes [35]. In addition, the ERK enzymes have been implicated in NK killing and LAK activation by IL-2 [30, 32, 35]. Although ERK-2 has been implicated in the lytic function of human NK cells, our results in NKL cells show that ERK2 does not appear to be involved in the immediate proliferation and activation produced by these agents. Moreover, ERK2 may be necessary but is not sufficient to produce these effects. The involvement of ERK2 may be more important in treatment with PSK. ERK3 expression might be involved in the proliferation and natural cytotoxicity of NKL cells induced by these agents. Our study also shows that p38 kinase activity is not required for IL-2 or PSK responsiveness in NKL cells. These results corroborate those obtained by Yu et al. [35] in NK cells activated by IL-2, but are different from those reported for T lymphocytes [3, 7].

The high expression of PKCβ and ERK2 in unstimulated NKL cells indicates that this isoform may be

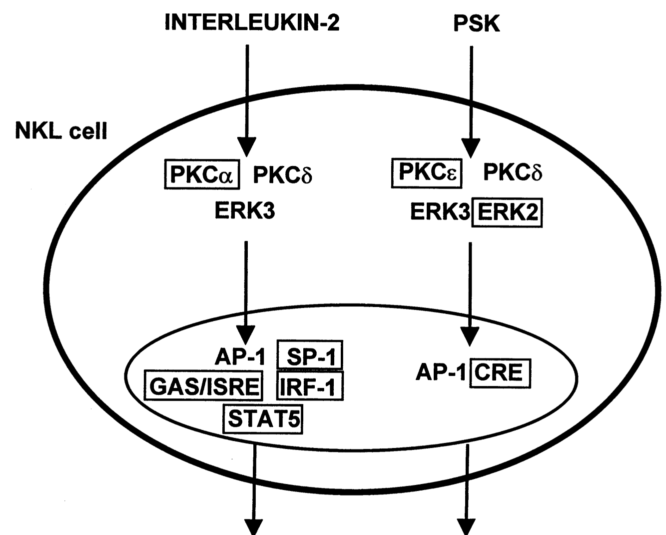

Fig. 3 Scheme of PKC isoenzymes, MAPK and binding activities possibly involved in signal transduction pathways stimulated by IL-2 and PSK on NKL cells

involved in the expression of different binding activities in unstimulated NKL cells [6]. PKCϵ might be responsible for the increase in CRE binding activity in PSK-treated NKL cells (Fig. 3). On the other hand, PKCα might induce the increase in SP-1 binding activity and the appearance of new complexes of GAS/ISRE, IRF-1 and STAT5 in IL-2-treated NKL cells (Fig. 3). The increase in AP-1 binding activity by PSK and IL-2 on NKL cells may be regulated by PKCδ and ERK3.

Our studies demonstrate that PSK and IL-2 induce different signaling pathways on NKL cells. Furthermore, we have demonstrated that the NKL cell line may serve as a good model for determining the biochemical and molecular events involved in these pathways. An understanding of these signaling molecular components may shed light on the mechanisms of action of PSK in cancer immunotherapy.

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