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Micro-encapsulated secretory leukocyte protease inhibitor decreases cell-mediated immune response in autoimmune orchitis

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ABSTRACT

Aims: We previously reported that recombinant human Secretory Leukocyte Protease Inhibitor (SLPI) inhibits mitogen-induced proliferation of human peripheral blood mononuclear cells. To determine the relevance of this effect in vivo, we investigated the immuno-regulatory role of SLPI in an experimental autoimmune orchitis (EAO) model.
Main methods: In order to increase SLPI half life, poly-ε-caprolactone microspheres containing SLPI were prepared and used for in vitro and in vivo experiments. Multifocal orchitis was induced in Sprague–Dawley adult rats by active immunization with testis homogenate and adjuvants. Microspheres containing SLPI (SLPI group) or vehicle (control group) were administered s.c. to rats during or after the immunization period.
Key findings: In vitro SLPI-release microspheres inhibited rat lymphocyte proliferation and retained trypsin inhibitory activity. A significant decrease in EAO incidence was observed in the SLPI group (37.5%) versus the control group (93%). Also, SLPI treatment significantly reduced severity of the disease (mean EAO score: control, 6.33 ± 0.81; SLPI, 2.72 ± 1.05). In vivo delayed-type hypersensitivity and ex vivo proliferative response to testicular antigens were reduced by SLPI treatment compared to control group ($p < 0.05$).
Significance: Our results highlight the in vivo immunosuppressive effect of released SLPI from microspheres which suggests its feasible therapeutic use.

Introduction

Secretory leukocyte protease inhibitor (SLPI) is an 11.7 kDa nonglycosylated protein originally identified in parotid gland secretions and in cervical, nasal and bronchial mucous. SLPI has been identified as a serine protease inhibitor with activity against cathepsin G, trypsin, and chymotrypsin, but primarily against neutrophil elastase (Saitoh et al., 2001). Expression of SLPI is induced in response to diverse inflammatory stimuli in various types of cells such as epithelial cells, neutrophils and alveolar macrophages (Saitoh et al., 2001; van Wetering et al., 2000a, 2000b; Williams et al., 2006). In the lung, SLPI is an alarm-mediating acute phase reactant secreted in response to LPS (Jin et al., 1998), interleukin-1, TNF-α (Sallenave et al., 1994), EGF (Velarde et al., 2005), defensins (van Wetering et al., 2000a, 2000b) and neutrophil elastase (Sallenave et al., 1994; van Wetering et al., 2000a, 2000b).

Strong evidence of widespread local production of SLPI in the male reproductive tract was shown by the presence of SLPI observed in seminal plasma from healthy volunteers (Ohlsson et al., 1995). SLPI expression was detected in epithelial cells of prostate glands, epididymis, seminal vesicles and the apical parts of germinal epithelium of the testis (Franken et al., 1989). The most likely the role of SLPI in the male and the female reproductive tract now appears to be a local protective function during inflammatory processes (Casslen et al., 1981; Wallner and Fritz, 1974).

SLPI has numerous functions unrelated to its protease-inhibitory activity since it may also function as an endogenous immunomodulatory, anti-inflammatory and/or anti-microbial substance (Fitch et al., 2006). By inhibiting IκB degradation, SLPI appears to have anti-inflammatory functions on leukocytes (Henriksen et al., 2004; Taggart et al., 2005; Xu et al., 2007). Also, SLPI reduces inflammatory gene expression and diminishes inflammatory cell accumulation (Lentsch et al., 1999; Ward and Lentsch, 2002). SLPI is able to upregulate macrophage production of anti-inflammatory cytokines (Sano et al., 2000). Also, it prevents release of pro-inflammatory cytokines by conjunctival epithelial cells (Seto et al., 2009). However, the in vivo effect of SLPI as an immunosuppressive agent has not been investigated in detail.

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The testis is considered an immune-privileged organ since it is able to tolerate auto-antigens expressed in germ cells. However, inflammation of the testis may occur and is frequently associated with infertility (Lustig and Tung, 2006). In fact, infection and inflammation of the male reproductive tract are widely considered important etiological factors of subfertility or infertility. Fifty percent of subfertile or infertile patients present different degrees of testicular lymphocyte infiltrates (Schuppe and Meinhardt, 2005).

EAO is a useful experimental model to study organ-specific autoimmunity and chronic testicular inflammation. EAO is characterized by a testicular interstitial cell infiltrate mainly composed of dendritic cells (Rival et al., 2007), macrophages (Rival et al., 2008) and lymphocytes (Jacobo et al., 2009), apoptosis and sloughing of germ cells from damaged seminiferous tubules resulting in aspermatogenesis and atrophy of seminiferous tubules (Doncel et al., 1989; Theas et al., 2003). In contrast with the immunosuppressive microenvironment characteristic of the normal testis, local secretion of pro-inflammatory cytokines has a major role in the induction of testicular inflammation (Guazzone et al., 2009).

It has been shown that is not efficient to administer SLPI intravenously or intraperitoneally since it is rapidly excreted by the kidneys (Bergenfeldt et al., 1990; Gast et al., 1990). To avoid this problem, we developed here a long-acting and controlled release mechanism for SLPI delivery. Biodegradable polyesters derived from lactic acid, glycolic acid and ε-caprolactone have been investigated for use in protein and peptide delivery (Hutchinson and Furr, 1989). The aim of the present study was to determine whether encapsulated SLPI might down-regulate testicular inflammation of rats with EAO. In our work we used poly-ε-caprolactone (PCL) microspheres containing SLPI as a controlled release system in order to increase its half life.

Methods

Animals

Adult male Sprague–Dawley rats aged 50–60 days were purchased from Bioterio Central, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (Buenos Aires, Argentina). Animals were kept at 22 °C with a 14 h light–10 h dark schedule and fed standard food pellets and water ad libitum. The experiments were performed in accordance with EC Directive 86/609/ECC and the Use and Care of Experimental Animals Committee of Facultad de Medicina, Universidad de Buenos Aires.

Production of recombinant human SLPI

Recombinant human SLPI (SLPI) was cloned and expressed as described previously (Maffia et al., 2007). Before using SLPI in in vivo and in vitro experiments, eluted fractions were purified with a polimixin B column. LPS contamination was <0.1 EU/μg protein as determined by the Limulus amebocyte lysate assay.

Preparation of microspheres containing SLPI

Microspheres of PCL (poly-ε-caprolactone) (Mw 14,000, Sigma-Aldrich, St. Louis, MO, USA) containing SLPI or not, were prepared using a water-in-oil-in-water (A1/O/A2) emulsion based solvent evaporation technique. Briefly, 400 mg of PCL were dissolved in 10 ml of dichloromethane (solution O, Anedra, San Fernando, Argentina) HPLC grade. One ml of a solution containing or not (control microspheres) 0.8 mg SLPI (A1) and 30 mg of mannitol was added to the oil solution (O) and mixed with a vortex for 1 min. This first emulsion (A1/O) was then added with constant stirring to 100 ml of an aqueous solution of 2% polyvinyl alcohol (A2, Riedel-de Haen, RDH, Seelze, Germany). The A1/O/A2 emulsion obtained was kept at room temperature (RT) for 4 h until complete evaporation of dichloromethane. Microspheres were then collected by centrifugation (5000 rpm at RT) and washed three times with distilled water. Finally, the microspheres were lyophilized.

Release kinetics studies

Thirty mg of dried microspheres were suspended in 300 μl of phosphate buffered saline at pH 7.4 and incubated at 4 °C and 37 °C for 40 days. Every five days, microspheres were centrifuged and supernatants recovered in order to evaluate free SLPI concentration. Afterwards, microspheres were resuspended with 300 μl of phosphate buffer and returned to the selected temperatures. The amount of SLPI released from microspheres was determined by sandwich ELISA.

SLPI inhibitory activity assay

Inhibitory serine protease activity of SLPI released from microspheres was evaluated by analyzing trypsin enzymatic activity. In brief, release SLPI (10 μl) was incubated with trypsin (5 μl, 8 mM, Gibco, Grand Island, NY, USA) for 10 min at RT in a 96-well plate. Then the colorimetric substrate of trypsin N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide (12 μg/ml, Sigma-Aldrich St. Louis, MO, USA) was added to each well. Hydrolysis of the substrate was measured by the spectrophotometric method at 405 nm in an ELISA plate reader for 50 min.

Induction of EAO

Rats were actively immunized with testicular homogenate (TH) prepared as previously described (Doncel et al., 1989). Briefly, rat testes were decapsulated, diluted in an equal volume of saline and disrupted in an Omni mixer for 30 s. The final concentration was 500 mg/ml wet weight. Rats were injected three times with 200 mg wet weight of TH/dose per rat at 14 days intervals. Antigen (0.4 ml) emulsified with 0.4 ml complete Freund's adjuvant (Sigma-Aldrich) was injected intradermally in footpads and at multiple sites near popliteal lymph nodes and the neck area. The first two immunizations were followed by an intravenous injection of 0.5 ml *Bordetella pertussis* (Bp) (strain 10536, Instituto Malbrán, Buenos Aires, Argentina) containing 10^{10} microorganisms and the third by intraperitoneal injection of 5×10^{9} microorganisms. Rats were killed 56 days after the first immunization. Blood was collected and sera stored at −70 °C until use. Popliteal, inguinal, renal and iliac lymph nodes (LN) and spleen were removed for cell proliferation assay. Testes were removed, weighed and processed as described below.

Experimental design

A group of rats were injected subcutaneously in the back and sides with 100 mg of microspheres containing SLPI in 500 μl saline solution with 1% carboxymethylcellulose and 0.1% Tween 20. Treatment started 48 h before the first immunization and continued throughout the experiment at 7 day intervals (treatment 1). For another group of rats treatment started 7 days after the last immunization (treatment 2). Control rats were immunized with TH and injected with empty microspheres (vehicle).

Histopathology

Testis histopathology was studied in paraffin-embedded Bouin's-fixed sections obtained from three different levels and stained with hematoxylin–eosin. To evaluate the degree of germ cell damage characteristic of EAO, we used a score described previously (Rival et al., 2008). Briefly, this score includes (a) percentage of damaged seminiferous tubules (ST), (b) degree of germ cell sloughing and (c) testicular/body weight ratio (T/Bw). Therefore, EAO score = V + T + P