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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

where V is the value assigned to the percentage of damaged ST (presenting germ cell sloughing and degeneration); V = 0 (0–3% damaged ST); 1 (3.1–4.9%); 2 (5–15.9%); 3 (16–25.9%); 4 (26–35.9%); 5 (36–55.9%); 6 (56–60.9%); 7 (61–79.9%); 8 (80–95.9%) y 9 (96–100%). T is an indicator of degree of germ cell sloughing: T = 0 when mild germ cell sloughing is observed and T = 0.5 when germ sloughing is severe (only spermatogonia and Sertoli cells are still attached to the ST walls). P is a correction factor corresponding to the T/Bw index: P = 0.5 when T/Bw index is less than 2.5×103.

Delayed-type hypersensitivity (DTH)

DTH was measured by a footpad swelling test performed at the end of each experiment. Rats were intradermically challenged in the left footpad with 2.10 mg of TH supernatant in 50 μl saline. This fraction was obtained by TH centrifugation (10,000 rpm at 4 °C). The other footpad was injected with the same volume of saline. Footpad thickness was measured with a micrometer 48 h after the challenge. Results of footpad swelling were expressed as mean of at least three measurements per rat (in mm), calculated as the difference between the thickness of the HT-injected footpad and the thickness of the saline injected footpad.

SLPI ELISA

A sandwich ELISA for SLPI was used to measure the protein. In brief, BD Falcon™ 96-well Clear ELISA Plates were coated overnight at 4 °C in coating buffer with 1 μg/ml mouse anti-human SLPI monoclonal antibody (Clone 20409 from R&D Systems, Minneapolis, MN, USA). Then, the plates were washed and non specific binding sites were blocked by incubating the wells with PBS-2% BSA in coating buffer for 30 min at 37 °C. Serially diluted SLPI standard and samples were then added and incubated overnight at 4 °C. The wells were then washed and polyclonal goat anti-human SLPI (Hbt, Uden, Netherlands) was added. After 90 min incubation at 37 °C, the wells were washed and peroxidase anti-goat IgG (Sigma, St Louis, USA) was added for 60 min at 37 °C. Then, 50 μl 3,3′,5,5′-tetramethylbenzidine (Abbott Diagnostics, Buenos Aires, Argentina) substrate solution was added and incubated for 5–30 min. The reaction was stopped with 50 μl of acidic stop solution and the plate was read at 450 nm.

In vitro cell proliferation assay

We used the [3H]-Thymidine incorporation assay to study activity of SLPI in cell proliferation. Cells from regional lymph nodes (LN, 5×10^5/well) were cultured in a 96-well plate with Concanavalin A (Con A 3 μg/well) or TH supernatant (55 μg/ml) in RPMI 1640 (Gibco, Grand Island, NY, USA), supplemented with heat inactivated 5% FBS (Gibco), 2 mM L-Glutamine, 0.05 mM 2-mercaptoethanol, 1 mM sodium pyruvate and 40 μg/ml gentamycin at 37 °C in a 5% CO_2 atmosphere. In some experiments, splenocyte proliferation was assessed in the presence of Con A + SLPI (2.5 μg/well). This concentration was chosen on the basis of previous results obtained with human peripheral blood mononuclear cells (PBMC).

Proliferation of human PBMC from healthy donors was assayed by treating cells with IL-2 (8 ng/ml) in the presence of microspheres (10 mg/well) added above a transwell (TW) bare 0.4-μm polycarbonate filter. Cell proliferation was determined on day five post-treatment with a pulse of 1 μCi/well of [3H]-thymidine (1 μCi/well, specific activity 5 mCi/mMol; Perkin-Elmer, Boston, MA, USA) for 18 additional hours and harvested on a multi-well cell harvester. Incorporation of radioactivity was measured in a liquid scintillation β-counter (1214 Rackbeta LKB Wallac, Perkin Elmer Instruments).

Statistical analysis

Comparisons of groups were assessed by the non-parametric Mann–Whitney test or post hoc Dunnett Multiple Comparisons Test as indicated in the legend of each figure. $P \leq 0.05$ was considered statistically significant.

Results

Trapped SLPI retained properties of soluble protein

SLPI inhibits human PBMC mitogen-induced proliferation (Guerrieri et al., in press). Rat SLPI coding sequences are known to be 75% homologous with human SLPI (Song et al., 1999). However, in order to assess whether SLPI also inhibits rat lymphocyte proliferation, 5×10^5 splenocytes were incubated in vitro with Con A (3 μg/well) in the presence or not of 2.5 μg/well of SLPI for 5 days as described in Methods. Fig. 1A shows that SLPI decreased splenocyte proliferation induced by Con A.

In order to determine the relevance of this effect in vivo, we assessed the immuno-modulatory effect of SLPI in rats with autoimmune orchitis. Since in vivo SLPI half life is very short (Bergenfeldt et al., 1990; Gast et al., 1990) we decided to use a method described in Methods by which SLPI was trapped in order to increase its half life. We first determined the ability of trapped SLPI to be released in vitro at 4 or 37 °C over a period of 40 days. Fig. 1B shows that SLPI was released at both temperatures in the first 24 h followed by a sustained release rate up to 21 and 30 days for 37° and 4 °C conditions, respectively. SLPI was detected at a higher concentration and for a longer period when microspheres were incubated at 4°, suggesting that higher temperatures may degrade the peptide faster. We also examined the inhibitory activity of released SLPI from microspheres by analyzing trypsin inhibitory activity (Fig. 1C). Eleven day culture supernatant from experiments at 4 °C and 37 °C shown in Fig. 1B was used to analyze inhibitory activity. As Fig. 1C shows, the 4 °C culture supernatant showed more inhibitory activity on trypsin than the 37 °C culture supernatant. SLPI biological activity was also tested on human lymphocyte proliferation. For this, 10 mg of microspheres with SLPI were placed above a transwell bare polycarbonate filter (0.4 μm pore size) while human PBMCs treated with IL-2 were added at the bottom of the transwell for 5 days. On day 6, proliferation was examined. Fig. 1D shows that SLPI release significantly inhibited lymphocyte proliferation, which suggests that this poly-ε-caprolactone microsphere system releases a biologically active protein.

Microspheres containing SLPI reduced the incidence and severity of EAO

Immunoregulatory activity of SLPI was studied in an experimental in vivo model of autoimmune orchitis. EAO was induced in male Sprague–Dawley rats as described in Methods. Two experimental designs were used to analyze activity of SLPI in vivo. In the first one (treatment 1), microspheres containing SLPI were administered before the first immunization; in treatment 2, microspheres were administered after the last immunization and continued until euthanasia. Histopathology showed that 93% of rats from the control group (EAO/vehicle treated) developed orchitis. In contrast, a significant decrease in EAO incidence was observed in rats injected with SLPI in both treatments (46% and 37.5% for treatments 1 and 2, respectively). Interestingly, we also found that SLPI significantly reduced severity of the disease compared to the control group ($p = 0.001$ versus treatment 1, $p = 0.035$ versus treatment 2) (Fig. 2A). This finding was confirmed by testicular histopathology showing that control group (EAO/vehicle treated) presented multifocal testicular damage characterized by an interstitial cell infiltrate composed mainly of monocytes, lymphocytes, macrophages and

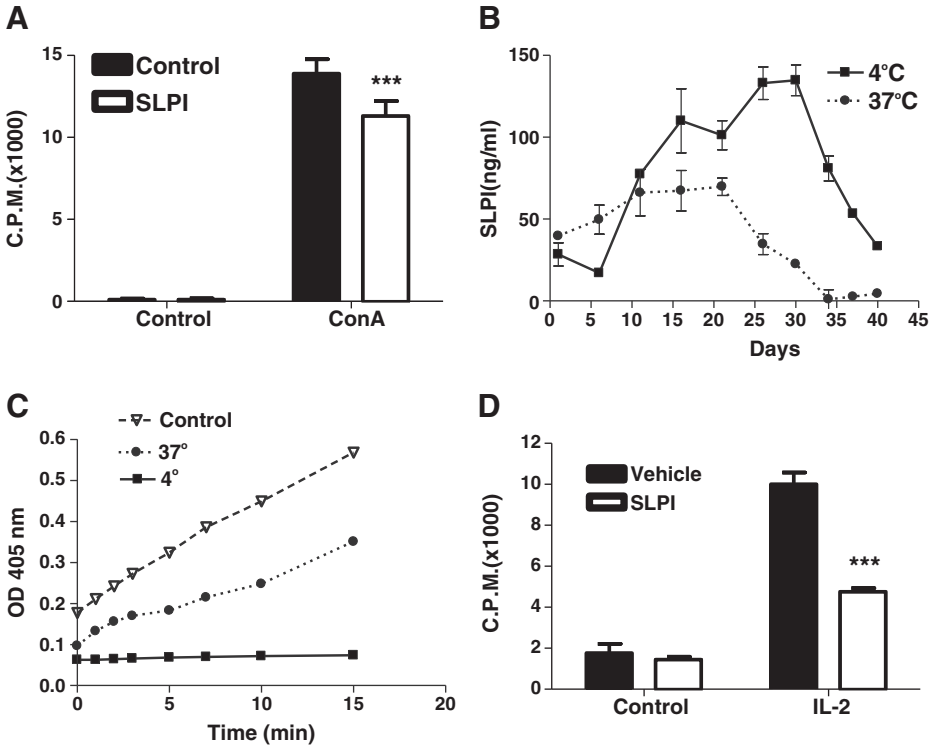

Fig. 1. (A) Effect of SLPI on cell proliferation. Splenocytes were seeded onto a 24-well plate stimulated with Con A 3 μg/well and incubated in the presence or not of SLPI 2.5 μg/well. Thymidine incorporation was measured on day 5 by 18 h pulse with [3H]-thymidine 1 μCi/well. Incorporation of radioactivity was measured in a liquid scintillation β-counter. Results are presented as mean ± S.E.M. of triplicate cultures from three experiments. (B) SLPI release kinetics demonstrated sustained release of SLPI at least over a period of 30 days. Thirty milligrams of dried microspheres were suspended in PBS and tubes were incubated at 4° and 37 °C. Samples were taken at specified time intervals for a total of forty days. The amount of SLPI released from microspheres was assayed by ELISAs. Results are presented as mean ± S.D. of triplicate from one representative experiment **(C)** SLPI released from microspheres inhibited trypsin protease activity. Samples from day 11 in 4 °C and 37 °C conditions were incubated with trypsin and the colorimetric substrate N-succinyl-Ala-Ala-Pro-Phe q-nitroanilide. Absorbance was monitored at 405 nm in a microplate reader. One representative experiment is shown (D) SLPI released from microspheres inhibited human lymphocyte proliferation. Cells were seeded onto 24-well plate stimulated with IL-2 (8 ng/ml) in the presence of SLPI microspheres (10 mg/well) or control microspheres separated with a 0.4-μm size polycarbonate filter. Thymidine incorporation was measured on day 5 by an 18 h pulse with [3H]-thymidine. Data are presented as the mean ± S.E.M. of triplicate cultures from two experiments. ***$p<0.001$ by ANOVA post hoc Dunnett Multiple Comparisons Test.

scarce neutrophils intermingled with Leydig cells and different degrees of germ cell sloughing of the seminiferous tubules (Fig. 2B). In contrast, most rats treated with SLPI presented little or no infiltrate and normal seminiferous epithelium with both treatments (Fig. 2C for treatment 2, and data not shown for treatment 1). Body weight of SLPI-treated rats did not differ from that of untreated normal rats suggesting that no apparent adverse effects were induced by SLPI treatments (body weight (g) mean ± S.E.M. of 13–16 rats/group: untreated normal rats, 472.3 ± 11.6; treatment 1, 456.4 ± 19.48 and treatment 2, 475.0 ± 12.77). Although both treatments prevented testicular pathology and decreased leukocyte infiltration, the experiments described below were done for the SLPI treatment 2 group in order to evaluate the immunoregulatory role of SLPI in the effector phase of the disease.

SLPI reduced cell mediated immunity in vivo and ex vivo

We next investigated effects of SLPI on T cell mediated immunity by analyzing in vivo DTH response and ex vivo LN lymphocyte proliferation to testicular antigens. Footpad challenge for DTH was tested in the treatment 2 group of rats 56 days after immunization. Fig. 3A shows that footpad swelling was significantly less prominent in rats treated with SLPI microspheres compared to control group (EAO/vehicle treated) ($p<0.05$) (Fig. 3A). LN were also recovered from SLPI or vehicle-treated rats and lymphocyte proliferation was assessed ex vivo in response to testicular antigens. Fig. 3B shows that lymphocyte proliferation of SLPI-treated animals was lower compared to vehicle-treated animals.

Discussion

In this study we demonstrated that SLPI can significantly inhibit testis damage. This effect of SLPI on EAO implies that SLPI inhibits multiple pathways either directly as a consequence of its antiprotease activity or via still undetermined regulatory pathways.

SLPI is secreted at the site of inflammation and presents anti-inflammatory activity. This anti-inflammatory activity has been demonstrated in eye and joint inflammation models in which several anti-inflammatory mechanisms were proposed such as the reduction of eosinophil and neutrophil recruitment, histamine release, suppression of NF-κB activation and the production of anti-inflammatory cytokines such as TGF-β and IL-10 (Murata et al., 2003; Sano et al., 2000; Sehnert et al., 2004).

The ability of SLPI to reduce autoimmune disease has been demonstrated in other animal models. Song et al. (1999) reported the effects of administration of SLPI to autoimmune arthritic rats and found that the overt tissue destruction and inflammation typically seen in this model could be suppressed by systemic injections of SLPI. Also, Mueller et al. (2008) studying the experimental model of myelin oligodendrocyte protein-induced experimental autoimmune encephalomyelitis (EAE) demonstrated the capacity of SLPI to increase proliferation of adult neural stem cell and oligodendroglial differentiation suggesting a novel role of SLPI in the promotion of tissue repair. Furthermore, expression of SLPI in macrophages, activated microglia, neuronal cells and astrocytes was upregulated more than one hundredfold during EAE attack. Recently, Wang et al. (2003) reported an up-regulation of SLPI in the brain of rats after ischemic stroke.

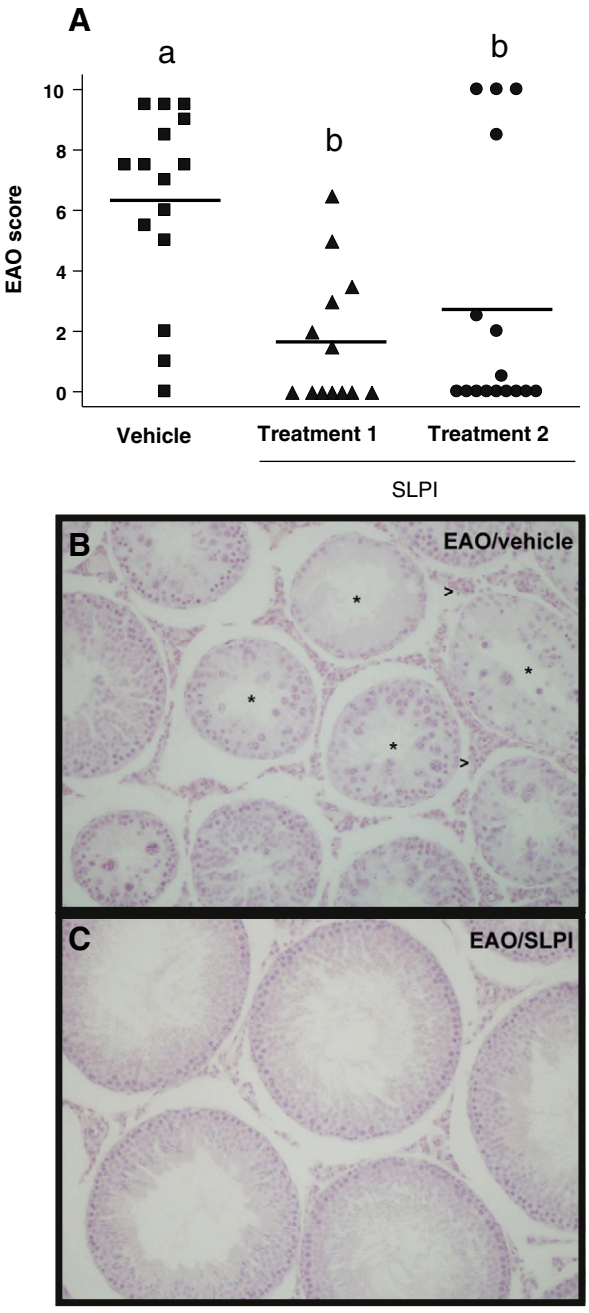

Fig. 2. Administration of SLPI reduced incidence and severity of EAO. (A) EAO score for rats immunized with TH and adjuvants and injected with empty microspheres (vehicle) or microspheres containing SLPI and euthanized 56 days after the first immunization. Similar EAO mean scores were observed in vehicle group with treatments 1 and 2. Horizontal lines represent the mean. Each symbol represents a single rat. Values with different letters superscript differ significantly ($p<0.05$, Mann–Whitney test). (B and C) Testicular histopathology from treatment 2 rats. Testis sections from EAO/vehicle (B) or EAO/SLPI (C) group rats sacrificed 56 days after the first immunization. EAO/vehicle rat presents a focus of testicular damage characterized by a moderate interstitial inflammatory cell infiltrate (>) and several seminiferous tubules (*) with different degrees of germ cell sloughing. In contrast, EAO/SLPI rat presents normal interstitium and seminiferous tubules. Note the severe tubular atrophy showing decreased diameter of seminiferous tubules in EAO/vehicle rat. H&E. Magnification 300×.

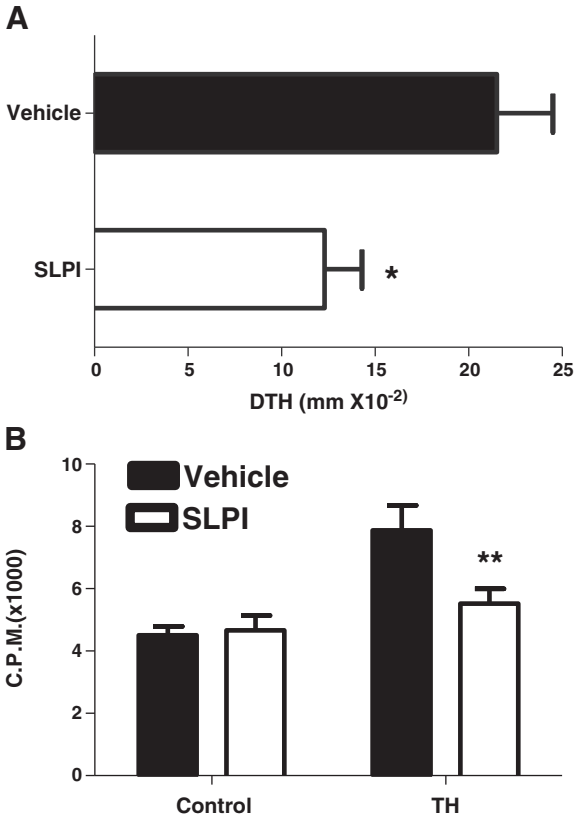

Fig. 3. (A) DTH is reduced by SLPI treatment. Rats with EAO treated with empty microspheres (vehicle) or microspheres containing SLPI were tested for DTH to testicular antigens. Two days before euthanasia (day 54), rats were injected with TH into left footpad. The right footpad was injected with saline. Footpad swelling was measured after 48 h and results are expressed as the mean difference between the thickness of the HT-injected footpad and the thickness of saline-injected footpad ± S.E.M. in each group. *$p<0.05$ (Mann–Whitney test). n = 16 rats/group. (B) SLPI controls' ex vivo cellular immune response. 5×10^5 cells obtained from lymph nodes of SLPI or vehicle-treated rats were cultured in microplates stimulated with 55 μg/ml testicular antigens (Ag). Thymidine incorporation was measured on day 5 by an 18 h pulse with [3H]-thymidine 1 μCi/well. Results are presented as mean ± S.E.M. from three independent experiments. **$p<0.01$ ANOVA post hoc Dunnett Multiple Comparisons Test. n = 16 rats/group.

Moreover, the administration of a recombinant adenovirus over-expressing SLPI into the cortical tissue resulted in a great reduction in ischemic lesion suggesting that ischemia-induced expression of SLPI might play a neuroprotective role in focal stroke.

The clinical application of SLPI is known to be limited due to rapid enzymatic cleavage by cathepsins and rapid clearance from the blood. The half life of an intravenous injection of SLPI is 120 min (Bergenfeldt et al., 1990). Approximately 80% of intraperitoneal injected SLPI (12 mg/kg) generated the maximal plasma concentration of 6 to 10 μg/ml 30 to 120 min after administration. When given intratracheally (8.6 mg/kg), SLPI was absorbed systemically, resulting in maximal plasma level of about 2 μg/ml 1 to 2 h after application and disappearing from the lungs with a half-life of 4 to 5 h (Stolk et al., 1995). Regardless of the route of administration, all studies suggest that the breakdown of the molecule occurs once it reaches the plasma. Therefore different formulations have been examined to increase the half life of this protein. For example, SLPI was encapsulated in 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine]: Cholesterol (DOPS: Chol) liposomes for inhalation. This formulation retained the anti-neutrophil elastase in in vitro studies (Gibbons et al., 2009). In our study, the formulation used was biocompatible both in vitro and in vivo.

Our study did not compare the effect of soluble SLPI versus micro-encapsulated SLPI. The reason we did not include this group of animals was based on previous and preliminary results obtained in our laboratory. They showed that a single administration of encapsulated SLPI, but not naked SLPI, five days before induction of foot pad inflammation was able to reduce swelling of mice feet (control $52\pm13.4\ mm\times10^{-2}$, encapsulated SLPI: $21\pm12.5\ mm\times10^{-2}$, naked SLPI: $48\pm6.2\ mm\times10^{-2}$). However, the administration of naked SLPI was

effective in reducing foot pad inflammation if the peptide was administered 5 h before induction of inflammation (SLPI 5 h: $28 \pm 16.1\ mm \times 10^{-2}$). These results suggest that administration of 'naked' SLPI would be effective in reducing acute but not chronic inflammation. The lack of effect of naked SLPI on inflammation caused 5 days later strongly suggests that naked SLPI would not have been effective in our orchitis model, at least for the second experimental design (i.e. rat treatment started 7 days after the last immunization). Therefore, we may speculate that PCL encapsulation of SLPI improves stability and potentially reduces the level and frequency of dosing required for therapeutic effect after s.c. administration.

The microspheres used have good permeability to proteins unlike other delivery systems such as polylactic acid (PLA) and polyglycolic acid (PGA) polymers. PCL degrades very slowly and does not generate an acid environment which can adversely affect the antigenicity of the peptide (Jameela et al., 1997). In the present study, we also observed that SLPI encapsulation did not modify anti-proteinase activity, suggesting that protein integrity was unaffected by encapsulation methods. However, the properties of temperature incubation modified the amount and activity of SLPI released: it was higher and more sustained when microspheres were incubated at 4 °C rather than at 37 °C. Based on these data, microspheres were administered every 7 days. It is important to mention that we were unable to detect blood SLPI concentration in rats in treatment groups 1 and 2. This could be due to: i) blood SLPI concentration below the detection limit of ELISA (1.25 ng/ml), ii) rapid degradation of the peptide; or iii) rapid uptake by cells and tissues. However, by concentrating the serum samples, SLPI serum concentration was detected in some animals (data not shown).

The delivery system used in our study was previously tested for protein delivery. It has been used for oral immunization, vaccines, mAb, bovine serum albumin, nerve growth factor, insulin and steroids (Sinha et al., 2004). To our knowledge this is the first time that PCL microspheres were used to deliver a serine proteinase inhibitor, demonstrating that encapsulation did not modify antiproteinase activity.

The precise mechanism by which SLPI treatment prevents EAO development is unclear. We may speculate that the mechanisms by which SLPI reduces EAO may operate in the induction as well as the effector phase of EAO. In fact, the effectiveness of SLPI in the second experimental designs and the reduction of DTH showed that the peptide acts at least in the effector phase. However, since SLPI was also effective for the first experimental protocol, we cannot discard an effect on the induction phase as well. We know that SLPI can target DCs and that it modulates multiple aspects of TLR mediated adaptive immune responses in mucosal draining LN (Samsom et al., 2007). If SLPI act on the induction phase of EAO, it may have direct extracellular interactions with TH. One possibility could be that human recombinant SLPI can bind to TH, thereby decreasing the possibility of immune cell recognition and interfering with activation of the transcription factor NF-kB by preventing degradation of inhibitory factor IkBα. However, the best known activity of SLPI is inhibition of leukocyte serine proteases. In fact, recombinant human SLPI inhibits murine serine proteases (Wright et al., 1999) and rat lymphocyte proliferation (Fig. 1A). We do not know whether the effect of SLPI on rat lymphocyte proliferation depends on antiprotease activity. However, preliminary results show that SLPI inhibits human lymphocyte proliferation an effect that does not depend on SLPI antiprotease activity (unpublished results). Further studies are needed to clarify whether reduction of EAO (incidence and severity) depends on SLPI serine protease activity inhibition.

Conclusion

Altogether, we demonstrate that it is feasible to use microencapsulated SLPI to directly reduce autoimmune damage. We also provide initial preclinical evidence to support use of this serine protease inhibitor to alleviate orchitis pathology. Based on our study and others, SLPI may represent a prototype of therapeutic approach for many autoimmune diseases. The data presented above describe for the first time that SLPI decreased EAO, even when the peptide is administered after the induction phase of the disease.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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