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Androgen-independent Effects of *Serenoa repens* Extract (Prostasan®) on Prostatic Epithelial Cell Proliferation and Inflammation

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Extracts from *Serenoa repens* are widely used for the treatment of benign prostatic hyperplasia (BPH) and traditionally for prostatitis. In the present study we evaluated the biological effects of *Serenoa repens* extract (Prostasan®) on prostate cells beyond its known antiandrogenic actions. Prostasan® inhibited epidermal growth factor (EGF) and lipopolysaccharide (LPS) induced proliferation of the prostatic epithelial, androgen independent cell line PC-3. At effective concentrations of 50 µg/mL, Prostasan® partly displaced EGF from EGF receptor (EGFR) but fully blocked EGF-induced cell proliferation of PC-3 cells. Similarly, Prostasan® inhibited LPS-induced proliferation of PC-3 cells without affecting LPS activation of the NFκB pathway via toll-like receptor-4 (TLR-4). Additionally, Prostasan® reduced the constitutive secretion of monocyte chemotactic protein-1 (MCP-1), the LPS-induced secretion of IL-12 and inhibited MCP-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) production in the presence of LPS on PC-3 cells. Taken together, our results suggest that *S. repens* extracts, in addition to other reported effects on BPH development and prostatitis, inhibits EGF-dependent growth and proinflammatory responses of the prostate epithelial cells. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: Serenoa repens; PC-3 cell line; BPH; proliferation; prostatitis; inflammation.

INTRODUCTION

Benign prostate hyperplasia (BPH) is a common form of enlargement of the periurethral mantel zone of the prostate leading to the benign prostatic syndrome (BPS) and a debated precursor of prostate cancer (De Marzo *et al.*, 1999). With increasing prostatic volume, obstructive problems are manifested in the form of delayed beginning of micturition, weak urinary stream and formation of residual urine in the bladder.

The enlargement of the prostate is based on increased cell growth and cell size (mainly of the fibromuscular stroma), but is also due to inflammatory processes resulting in compression of the urethra. Inflammatory infiltrates are frequently observed in resection and biopsies from patients with enlarged prostates (Di Silverio *et al.*, 2003; Robert *et al.*, 2009), and correlation between urinary symptom severity and intraprostatic inflammation has been proposed (Robert *et al.*, 2009). Although the exact mechanisms of inflammation in relation to prostate growth are not yet fully understood, activation of macrophages or lymphocytes and secretion of cytokines appear to be important (St Sauver and Jacobsen, 2008). Therefore, once inflammation is initiated, chemotactic mediators such as interleukin-8 (IL-8) or monocyte chemotactic protein-1 (MCP-1) lead to further cellular infiltration and their subsequent activation by interferon (IFN) γ, IL-2, IL-4 and IL-6 (Royuela *et al.*, 2004; Penna *et al.*, 2007). As well as macrophages, prostatic epithelial cells are able to secrete proinflammatory cytokines, thus contributing to the inflammatory process (Di *et al.*, 2009; Pei *et al.*, 2009).

Conventional treatment of BPS aims to reduce cellular proliferation by inhibiting the function of the 5-alpha-reductase, an enzyme that converts testosterone into dihydrotestosterone (Finasteride), and/or to lower the prostatic smooth muscle tonus by selective alpha-blockers (Doxazosin) (Tacklind *et al.*, 2009). Also, extracts made by lipophilic extraction from the dried berries of saw palmetto (*Serenoa repens*) are commonly used for the treatment of BPH and prostatitis (Wilt *et al.*, 2002; Hong *et al.*, 2010). Although the mechanism is not yet completely understood, several pharmacological mechanisms have been proposed for different compositions of saw palmetto extracts including: antiproliferative (MacLaughlin *et al.*, 2006) and antioedematic effects (Fagelman and Lowe, 2001); inhibition of the 5-alpha-reductase (Délos *et al.*, 1994; Bayne *et al.*, 1999; Pais, 2010) and 5-lipoxygenase (Paubert-Braquet *et al.*, 1997); reduction of eicosanoid (Paubert-Braquet *et al.*, 1997) and epithelial growth factor (EGF) production (Di Silverio *et al.*, 1998); and binding to the α1-adrenergic receptor (Suzuki *et al.*, 2007) among others.

However, several questions about the biological actions remain to be elucidated since *S. repens* does not display the typical side effects of drugs specifically

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designed to inhibit testosterone metabolism, such as ejaculation disorders, gynecomastia or the reduced production of prostate-specific antigen (PSA; Wilt *et al.*, 2002; Habib *et al.*, 2005). Considerable research has been carried out using *S. repens* concentrations higher than 100 µg/mL and it is possible that biological effects of *S. repens* in therapeutic doses are exerted through pathways that are unrelated to androgen actions (Bayne *et al.*, 1999; Fagelman and Lowe, 2001; Suzuki *et al.*, 2007). As a number of studies have shown clinical effects of *S. repens* we consider it very important to further explore the mechanism responsible as this can help us to better use this drug and to form a basis for further therapeutic developments. Therefore, the present study aimed to investigate the effects of lipophilic extract of *S. repens* (Prostasan®) on alternative proliferation inducers of prostate cells as well as on its antiinflammatory potential at physiologically relevant concentrations lower than 100 µg/mL (Bayne *et al.*, 1999).

MATERIAL AND METHODS

Test substance. We investigated a *S. repens* lipophilic extract (Prostasan®) that was provided by A. Vogel Bioforce AG (Roggwil, Switzerland). Prostasan® was prepared using a drug extract ratio of 9–12:1 and 96% (v/v) ethanol as extracting agent. According to the certificate of analysis, batch 8828 was characterized by 86% total content of fatty acids.

Receptor binding studies. Membranes of A431 cells naturally expressing EGF receptors were incubated with 100 pM [^{125}I]-hEGF and increasing concentrations of Prostasan® for 1 h at 25°C. After filtration the filter-bound radioactivity was measured by a scintillation counter. Non-specific radioligand binding was defined in the presence of 100 nM hEGF. The inhibitory concentration IC_{50} was determined by sigmoidal dose–response algorithms to identify the amount of extract required to displace 50% of the natural ligand from the receptor.

Cell Culture. PC-3 cells were cultured in RPMI 1640 supplemented with 10% foetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). Cells were grown in the presence of 5% CO_2 at 37°C. In order to starve the cells, RPMI 1640 supplemented with 5% FBS stripped of steroids with Dextran coated charcoal (DCC-FBS) and antibiotics was used.

Cytokine Measurements. Cells were seeded in normal medium; after 24 h cells were treated with the different Prostasan® concentrations, and 1 h later cells were supplemented with 1 µg/mL lipopolysaccharide (LPS) derived from *Escherchia coli* O26: B6 (Sigma-Aldrich, St Louis, USA). Cell media were collected after 1, 2 and 4 days post-treatment and analysed for the presence of cytokines with the MAP Human Cytokine/Chemokine premixed 14-plex kit (Millipore, Billerica, MA) on a Luminex instrument. Experiments were performed in triplicate. Fourteen different cytokines were measured (GM-CSF, IFN- γ, MCP-1, TNF-α, IL-1beta, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13). The levels of TNF-α, IL-2, IL-4, IL-5, IL-13 were below the detection limit (data not shown).

Proliferation assay. Cell proliferation was measured with the xCelligence system (Roche Diagnostics, Indianapolis, IN, USA). 2000 cells were first seeded in normal medium per well; after 24 h the medium was changed to starvation medium. 20 h later the cells were treated with EGF, LPS, Prostasan® or a combination thereof as specified in the figure captions and observed for at least 4 days. Automatic measurements were performed every 15 min for the duration of the experiment.

Data analysis and statistics. The xCelligence system employs 96 well plates with gold electrodes at the bottom of the wells. It detects the proliferation of adherent cells by recording the change in impedance of the gold electrode at the bottom of the well that an increase in the number of cells causes. The technique was described earlier (Tan *et al.*, 2009). The data are presented in the form of a unitless variable termed the *Cell Index* (CI). It is calculated from the following formula:

$$Cell\ Index = (Z_i – Z_0)/15$$

where Z_i is the impedance at timepoint i and Z_0 is the impedance of the medium measured at the start of the experiment. Data are presented as Change in Cell Index (ΔCell Index) ± standard error measurement (SEM), for measurements made every 24th hour. The number of replicates is specified in the figure captions.

The statistical analysis was performed using Graphpad Prism 5.0 (Graphpad Software Inc., La Jolla, CA, USA). In Figs 1A, 1B and 2 linear regression of the log-transformed Cell Index values was performed in order to investigate the effect of Prostasan® LPS and EGF on the growth rate of the cells. The calculated slopes were compared with a two-tailed test to investigate if the observed differences in growth rate were significant. For all three experiments the slopes were compared with the sample not treated with Prostasan® (PO). In Fig. 3 a two-tailed student's *t*-test was employed to investigate if the observed differences were statistically significant. Differences were considered statistically significant if the probability that they occurred by chance was <0.05.

RESULTS

Prostasan® inhibition of prostate epithelial cell proliferation and antagonism with EGF receptor

To study the effects of the *S. repens* extract (Prostasan®) on EGF actions in prostate epithelium, independently from androgen activity, we used the androgen receptor (AR) negative/androgen insensitive prostate cancer PC-3 cells as a model (Kaighn *et al.*, 1979). First, PC-3 cells were incubated with increasing concentrations of EGF to evaluate their proliferative responsiveness. For further studies 100 ng/mL was defined as an appropriate concentration (data not shown).

Next, we analysed the specific effect of Prostasan® on EGF-induced proliferation. PC-3 cells were pretreated with increasing concentration of Prostasan® for 1 h prior to the addition of EGF and then incubated for 5 days. At 50 µg/mL Prostasan® effectively inhibited

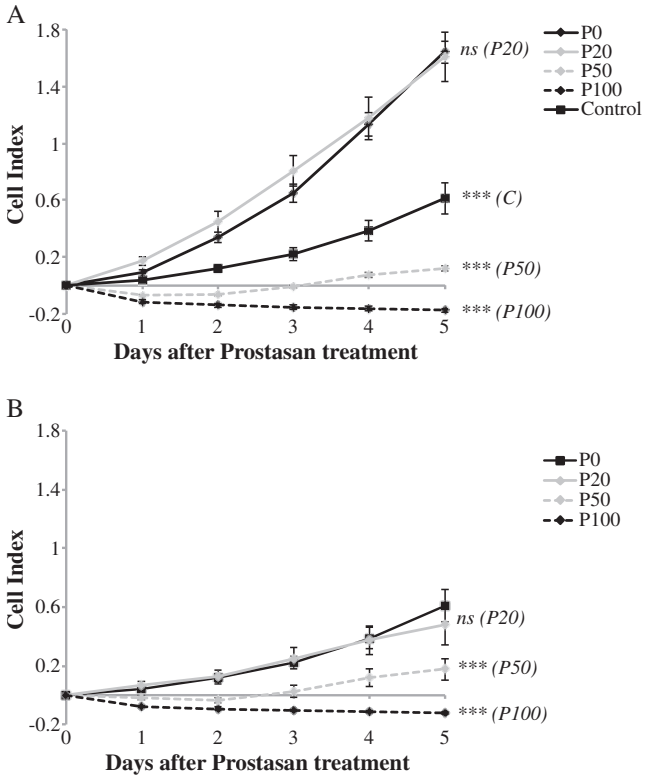

Figure 1. Effects of epithelial growth factor (EGF) and *Serenoa repens* extract (Prostasan®) on the proliferation of PC-3 cells. (A) PC-3 cells were incubated for 1 h with 0, 20, 50 and 100 µg/mL of Prostasan® then stimulated with 100 ng/mL of EGF and the proliferation was measured over time (P0, P20, P50 and P100). Data from normally growing and untreated cells are denoted control. The mean of two triplicate experiments is shown and the standard error measurement (SEM) is indicated. Data were analysed by log-transformation and linear regression. Significant differences in growth rate compared with P0 are indicated in the graph (ns = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). (B) Proliferation of PC-3 cells incubated with Prostasan® as in (A) and proliferation measured overtime in the absence of EGF. The mean of two triplicate experiments is shown, except for P20 where $n = 5$, and the SEM is indicated. Data were analysed by log-transformation and linear regression. Significant differences in growth rate compared with P0 are indicated in the graph (ns = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

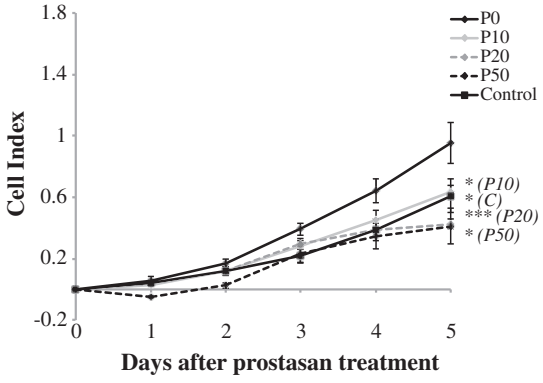

Figure 2. Effects of lipopolysaccharide (LPS) and Prostasan® on PC-3 cells proliferation. PC-3 cells were incubated for 1 h with 0, 10, 20 and 50 µg/mL of Prostasan® then stimulated with 1 µg/mL of LPS and the proliferation was measured overtime (P0, P10, P20 and P50). Data from normally growing and untreated cells are denoted control. The mean of two duplicate experiments is shown, except for P0 where $n = 3$, and the standard error measurement (SEM) is indicated. Data were analysed by log-transformation and linear regression. Significant differences in growth rate compared with P0 are indicated in the graph (ns = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

cell growth, thus reducing the proliferative effects of EGF (Fig. 1A). Concentrations of <20 µg/mL of Prostasan® did not inhibit EGF-induced growth of PC-3 cells, whereas concentrations ≥100 µg/mL completely abolished cell growth. In consonance with previous reports (Petrangeli *et al.*, 2009), in the absence of EGF stimulation, *S. repens* extracts inhibited cell proliferation at concentrations of >20 µg/mL (Fig. 1B).

In an attempt to further understand how Prostasan® exerts its inhibitory action, we investigated its capacity to bind to the human EGF receptor (EGFR). The specific displacement of radiolabeled hEGF from the human recombinant EGF receptor by Prostasan® is given in Fig. 4. Partial competition was observed as low as at 50 µg/mL and the calculated concentration at which the extract displaced 50% of the natural ligand, IC_{50}, was 71.2 µg/mL. A Hill coefficient of −2.77 indicates that *S. repens* extract bind to hEGFR in a multifactorial process and not as a monosubstance binding to a specific binding cleft.

Together these results show that *S. repens* extract inhibits EGF-induced proliferation of PC-3 cells. Since only partial occupancy of the EGFR occurs at the concentration of 50 µg/mL but complete inhibition of proliferation takes place, this suggests that in addition to the specific receptor blockage further pathways downstream of the receptor binding could be influenced by the extract.

Actions of LPS and Prostasan® on PC-3 cell proliferation

Lipopolysaccharide, the major component of the Gram-negative bacteria cell wall is commonly used to study inflammatory responses in cells. Lipopolysaccharide, in addition to its inflammatory actions, seems to activate proliferation of prostate cells (Kundu *et al.*, 2008). Therefore, we investigated the effects of 1 µg/mL LPS on the growth of PC-3 cells over 5 days. The result showed a weak but significant effect of LPS on cell proliferation (Fig. 2). Even low concentrations of Prostasan® (10 µg/mL) reduced the proliferation induced by LPS. Higher concentrations of Prostasan® (50 µg/mL) markedly suppressed cell growth to levels similar to untreated cells.

These effects can be explained by interference of binding of LPS to its cognate Toll-like receptor (TLR-4) or by interference with TLR-4 signalling pathways. To this end we investigated the potency of Prostasan® to inhibit transcription of the NFκB reporter gene in the TLR-4 expressing HEK cell line (HEK-Blue™ hTLR, Invivogen, Toulouse, France). Prostasan®, at concentrations between 10 and 250 µg/mL, did not reduce transcription of the reporter construct, suggesting that Prostasan® does not interfere with the NFκB pathway induced by LPS (data not shown).

Prostasan® effects on IL-12, GM-CSF and MCP-1 secretion from PC-3 cells

Lipopolysaccharide is able to induce an inflammatory reaction in the responsive tissue by inducing the production of cytokines. Therefore, we investigated the effects of Prostasan® on the PC-3 cells cytokine

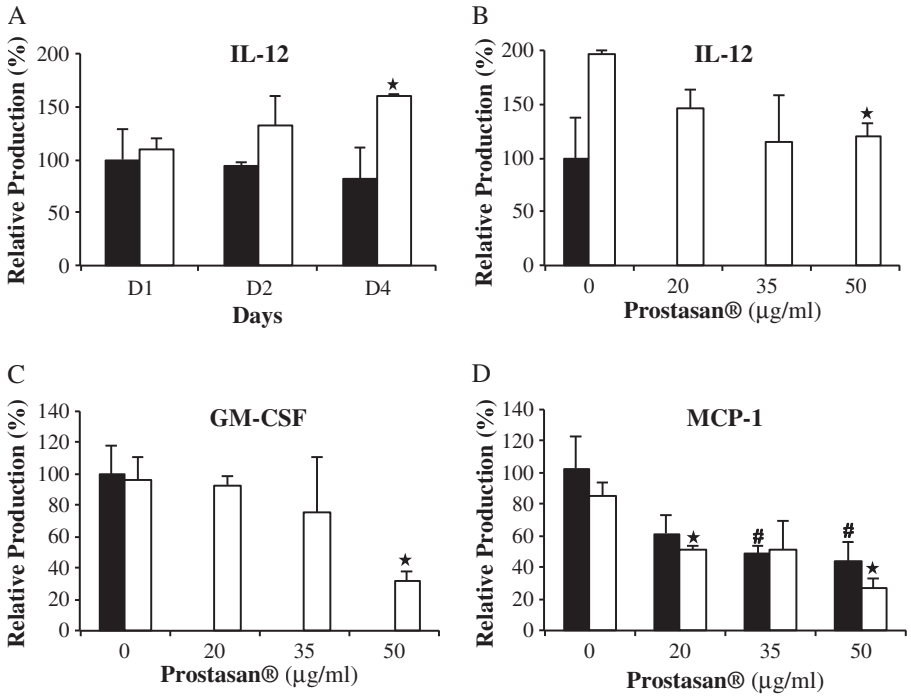

Figure 3. Effect of lipopolysaccharide (LPS) and Prostasan® on the cytokine secretion from PC-3 cells. (A) Secretion of interleukin (IL) 12 from PC-3 cells treated with 1 µg/mL LPS (white bars) or vehicle (black bars) for 1 (D1), 2 (D2) and 4 days (D4). Data are expressed as relative concentration of IL-12 compared with the detected in the conditioned medium of vehicle-treated cells at day 1. Statistical evaluation of the mean was performed by two-tailed Student's *t* test comparing each condition with its control at day 1 (* = $p < 0.05$). Relative concentration of (B) IL-12 and (C) granulocyte-macrophage colony-stimulating factor (GM-CSF) in the conditioned medium of PC-3 cells incubated with LPS and the indicated concentration of Prostasan® for 4 days. Data are shown as relative concentration, where the concentration of IL-12 and GM-CSF in the conditioned medium of vehicle-treated cells at day 4 (black bar) was set to 100%. Two-tailed Student's *t* test was used to analyse the statistical significance of the effect of each Prostasan® concentration on the cytokine release compared with the non-Prostasan® treated control (* = $p < 0.05$). (D) Secretion of monocyte chemotactic protein (MCP) 1 from PC-3 cells incubated with (white bars) or without (black bars) 1 µg/mL LPS and the indicated concentration of Prostasan® after 4 days. Data are shown as relative concentration, where the concentration of MCP-1 in the conditioned medium of vehicle-treated cells was set to 100%. Two-tailed Student's *t* test analysis was used to compare the effect of each Prostasan® concentration with untreated control (* = $p < 0.05$; # = $p < 0.07$).

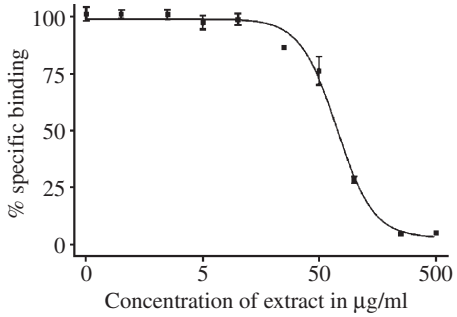

Figure 4. Human epithelial growth factor (EGF) receptor expressing A431 cells were incubated with radioactively labeled [^{125}I]-hEGF in the presence of increasing doses of *Serenoa repens*. Receptor-bound radioactivity was measured by scintillation counter. The specific binding in percentage (mean ± SEM) of duplicates is illustrated.

secretion in response to LPS overtime for 4 days. Fourteen different cytokines were measured (GM-CSF, IFN-g, MCP-1, TNF-a, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13). Of the measured cytokines, only IL-12 showed a significant and time dependent induction by LPS (1.6-fold after 4 days; Fig. 3A). LPS induction of IL-12 was significantly reduced (40%) to basal levels by co-incubation with 50 µg/mL of Prostasan® (Fig. 3B), while no significant effects were observed on the constitutive production of IL-12 by Prostasan® alone. Additionally, in the presence of LPS, Prostasan® (at 50 µg/mL) inhibited the production of GM-CSF by 70% (Fig. 3C). Similar to IL-12, Prostasan® did not affect the constitutive production of GM-CSF significantly (data not shown). On the other hand, Prostasan® inhibited MCP-1 secretion in LPS-treated PC-3 cells at 20 and 50 µg/mL (showing a trend at 35 mg/mL) by 50 and 75% respectively and in non-LPS-stimulated cells at concentrations of 35 and 50 µg/mL by 50% (Fig. 3D). An inhibitory trend on the LPS-induced IL-8 production was also observed. However, no definitive conclusions may be addressed for IL-8 production since in the absence of Prostasan® the signal was above the detection limit but returned to 30% below this value in the presence of Prostasan® at 50 µg/mL (data not shown). Finally, Prostasan® did not affect IL-7 and IL-10 production.

DISCUSSION

In this work we investigated the inhibitory effects of *S. repens* extract Prostasan® on the epithermal growth factor (EGF) and the Gram-negative bacteria cell wall component LPS-induced proliferation of prostatic cells PC-3 (Figs 1 and 2). In the case of EGF, the mechanism of Prostasan®'s inhibitory action involves competition for the EGF receptor (Fig. 4). Since EGF plays a central role in BPH and prostate cancer, including late stages of androgen-independent prostate cancer (Marengo and Chung, 1994; Festuccia *et al.*, 2005), the interference

with EGF actions might represent a possible alternative or complementary treatment to both prostatic disorders. Naturally, further studies *in vivo* are required to support the *in vitro* evidences. On the other hand, LPS exerts a milder but still significant induction on proliferation of prostate cells, supporting the role of bacterial infection in the origin of BHP, which would be inhibited by treatment with *S. repens* extracts. Together, these findings support the notion that *S. repens* extracts inhibit prostate growth at different levels such as androgen dependent and independent proliferation by interfering with the function of the 5-alpha-reductase (Bayne *et al.*, 1999; Raynaud *et al.*, 2002; Hill and Kyprianou, 2004; Habib *et al.*, 2005), altering the cell membrane organization (Petrangeli *et al.*, 2009) and competing with the ligands binding to the receptor (Fig. 4). The mechanism behind *S. repens* extract effecting LPS actions on prostatic cells remains to be elucidated since they do not apparently affect Toll-like receptor 4 (TLR4) intracellular signalling. Yang and colleagues reported that STAT-3 phosphorylation is affected by *S. repens* in prostatic cells (Yang *et al.*, 2007). As phosphorylation of STAT-3 is sensitive to LPS, this might represent a possible point of action for Prostasan®. However, further studies are needed to fully understand the mechanisms behind the influence of Prostasan® on prostatic cell growth and inflammation.

Lipopolysaccharide also triggers an immune response leading to proinflammatory cytokine secretion from activated macrophages in the prostate environment in a process inhibited by *S. repens* extracts (Permixon®) (Bonvissuto *et al.*, 2011). However, cytokine production in response to bacterial infections is not restricted to cells of the immune system (Di *et al.*, 2009). In fact, prostate cells express Toll-like receptors and respond to LPS stimulation (Fig. 3; (Pei *et al.*, 2009), which indicates that prostate cells themselves contribute to the inflammatory reaction. Prostasan® inhibits this process (Fig. 3).

One of the cytokines induced by LPS in PC-3 cells isIL-6 (Pei *et al.*, 2009), which plays a major role in the inflammatory process in the prostate (Rodríguez-Berriguete *et al.*, 2010), however, in our experiments we did not detect such induction, possibly due to the constitutive high levels of basal expression from unstimulated cells (~850 pg/mL) we observed. Remarkably, IL-12 was the only cytokine induced by LPS in PC-3 cells. Induction of IL-12 by LPS is commonly seen in activated macrophages as part of the inflammatory reaction, but to our knowledge it has not been reported previously in PC-3 cells (Fig. 3). Therefore, constitutive and induced production of IL-12 from epithelial prostatic cells would contribute to the inflammatory process during BPH and might collaborate in the transition to a malignant stage.

Additionally, PC-3 cells constitutively secrete monocyte chemotactic protein-1 (MCP-1), a cytokine involved in prostatic growth dysregulation and benign prostatic hyperplasia (Fujita *et al.*, 2010). Prostasan® inhibits constitutive MCP-1 secretion from PC-3, which would directly affect these processes. Finally, the role of GM-CSF in the pathogenesis of benign prostatic hyperplasia is less well understood although this factor is a general mediator of immune processes and inflammation. Taken together, our data suggest that treatment with *S. repens* extracts, which inhibit IL-12, MCP-1 and GM-CSF secretion, would limit the contribution of the prostate epithelial cells to the inflammatory processes, in addition to the effects on the production of proinflammatory cytokines from macrophages in BPH development, and therefore would lead to a symptomatic relief and, maybe, prevention of malignant transformation in the prostate.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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