THE secretory leucocyte proteinase inhibitor (SLPI) is a low molecular weight, tissue-specific inhibitor of, for example, elastase and cathepsin G, which also have antimicrobial capacity. SLPI has been localised to the respiratory, gastrointestinal and genital tracts, but so far not to the kidney. The presence of SLPI in renal tubuli cells was demonstrated using immunohistochemistry and, by means of *in situ* hybridisation on human renal biopsies, we were able to demonstrate SLPI production. In various inflammatory conditions in the kidneys, the protease-antiprotease balance is disturbed. For this reason, as well as the possible role in the defence against ascending urinary tract infections, it is interesting to establish a source of SLPI in renal tubuli cells.

Key words: Glomerulonephritis, Kidney, Protease inhibitor, SLPI, Anti-leukoprotease

Novel distribution of the secretory leucocyte proteinase inhibitor in kidney

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Introduction

Secretory leukocyte proteinase inhibitor (SLPI) is a 107 amino acid non-glycosylated single chain protein, stabilised by intrachain disulphide bonds. The estimated molecular weight of the acid-stable protein is 12 kDa. The C-terminal is responsible for the inhibitory activity against elastase, cathepsin G, chymotrypsin and trypsin, whereas the N-terminal has been reported to possess antimicrobial capacity.[1,2] SLPI is also known to inhibit HIV type 1 by blocking DNA synthesis.[3] The Kazal-type inhibitor forms complexes with the proteases and has a half-life of 10 min in the circulation. The complexes formed locally dissociate in plasma and the proteases are taken over by the plasma protease inhibitors (alpha-1-antitrypsin, alpha-2-macroglobulin and antichymotrypsin).[1] Furthermore, SLPI has been shown to inhibit elastin-bound elastase, which may be of importance for the preservation of elastic lung tissue.[4] The inhibitor is also capable of upregulation of macrophage production of the anti-inflammatory cytokines interleukin-10 and transforming growth factor-β.[5] The SLPI gene contains binding sites for transcription factors induced by, for example, tumour necrosis factor-α, and an upregulation of SLPI is seen in several inflammatory disorders.[6] The physiological functions of SLPI are probably to buffer the extracellular protease-mediated effects of inflammatory leukocytes, as well as participating in the antimicrobial defence. SLPI has until now mainly been found in mucosa (e.g. in the respiratory, gastrointestinal and genital tracts). Less is known about SLPI in solid organs, although production was recently established in human pancreatic β cells, indicating an autocrine/paracrine function.[7]

The aim of the study presented here was to clarify whether there is a local renal production of SLPI. A possible local production would be of great interest, considering the multitude of inflammatory conditions, including different kinds of glomerulonephritis and nephritis, involving the kidneys. Anti-neutrophilic cytoplasmic antibodies (ANCA) are autoantibodies directed against constituents of polymorphonuclear neutrophils and monocytes, associated with systemic vasculitis and glomerulonephritis. One of the ANCA antigens, proteinase 3, has the capacity to attack and degrade components of the extracellular vessel wall matrix and the glomerular basement membrane. In blood, it is immediately bound, mainly by alpha-1-antitrypsin. Patients with ANCA-associated vasculitis have an over-representation of the PiZ gene of alpha-1-antitrypsin and thus a lack of the inhibitor.[8] SLPI has no direct inhibitory capacity against proteinase 3; it can, on the contrary, be degraded proteolytically by it.[1] Although SLPI has no direct inhibitory capacity against proteinase 3, the progressive binding of elastase to SLPI instead of alpha-1-antitrypsin has been shown to be paralleled by an increasing binding of

proteinase 3 to alpha-1-antitrypsin.[9] With this in mind, SLPI may indirectly aid in the binding and inhibition of proteinase 3. SLPI is also an inhibitor of the production of matrix metalloproteinases (MMP) and, knowing that MMP expression and activity are greatly elevated in inflammatory disorders of the kidney, a possible renal production would be very interesting.[10,11] A possible role in the defence against ascending urinary tract infections also draws attention to the point of issue.

Subjects and methods

Patient material

Macroscopically and microscopically normal kidney preparations were selected from three different specimens obtained at the time of nephrectomy due to renal cancer. The Helsinki declaration regarding the use of human tissues was strictly observed.

Materials and chemicals

Normal rabbit serum was purchased from Dakopat AB (Copenhagen, Denmark). Swedish landrace goats were immunised with recombinant human SLPI, to produce anti-SLPI antiserum. Biotinylated rabbit anti-goat IgG and avidin-biotinylated horseradish peroxidase complexes were from Vector Laboratories (Burlingame, CA, USA). The AEC substrate-chromogen system and Faramount aqueous mounting medium were from Dako Corporation (Carpinteria, CA, USA). An oligonucleotide probe cocktail for SLPI was from R&D systems (Abingdon, UK). Blocking reagent and anti-digoxigenin (DIG)-AP conjugate (Fab fragments) were from Boehringer Mannheim (Mannheim, Germany). Visualising substrates (bromochloroindolylphosphate (BCIP) and nitroblue tetrazolium (NBT)) were from Bio-Rad Laboratories (Hercules, CA, USA).

Immunohistochemistry

The frozen renal tissue specimens were fixed in 4% buffered formaldehyde, dehydrated and embedded in paraffin wax for sectioning on sialinised slides. After rehydration, the sections were exposed to pepsin (4 mg/ml in 0.01 M HCl) for 20 min at 37°C. The sections were then washed between every step in Tris-buffered saline (TBS), 3×5 min. Endogenous peroxidase activity was removed by incubation of the specimens in 0.3% H_2O_2 in methanol for 30 min at room temperature. Normal rabbit serum (3% in TBS) was applied to block non-specific staining. The next step, after draining, was to incubate the specimens with primary antibody (goat anti-SLPI, 1/1000, 30 min). For detection, we used biotinylated rabbit anti-goat IgG (5 µg/ml), followed by application of avidin-biotinylated horseradish peroxidase complexes

for 30 min. Visualisation was accomplished by addition of AEC (0.75 mg/ml of 3-amino-9-ethylcarbazole in 2.5% *N,N*-dimethylformamide and 50 mM acetate buffer) that was left to incubate for 15 min. The slides were washed and counterstained before mounting. All the incubations were carried out at room temperature.

Adsorbed anti-SLPI antiserum, obtained by affinity chromatography on a SLPI-conjugated Sepharose 4B-column, was used as a negative control and applied instead of the primary antibody.

In situ hybridisation

A cocktail of three 30-base long oligonucleotide probes (5′-TCTTAGGAGGACAGACTC CAGCTTT-GAAGG-3′, 5′-ATTTCCCACACATGCCCATG CAA-CACTTCA-3′, 5′-AAC ATCTCTTCTTCCCTGGA-CACTGCCAGT-3′), based on the antisense sequence of SLPI and labelled with DIG at the 5′ end, was used for the *in situ* hybridisation. Diethylpyrocarbonate was added to the millipore water and all solutions to block RNAse activity. All solutions were autoclaved or sterilised by filtration.

Renal tissue was taken immediately after surgery, put into liquid nitrogen and stored at –70°C. After sectioning on sialinised slides, the specimens were left to dry in air. After fixation (4% paraformaldehyde in $1 \times$ phosphate-buffered saline (PBS), 2 h, 4°C), the slides were incubated in a sucrose solution (30% sucrose in $1 \times$ PBS, overnight, 4°C). Washing in $1 \times$ PBS for 2×5 min was followed by treatment with 0.1 M glycine, 2×5 min. After further washing in $1 \times$ PBS for 2×5 min, the sections were placed in 0.3% Triton X-100 in $1 \times$ PBS for 15 min. Washing in $1 \times$ PBS, 2×5 min, was followed by incubation with proteinase K (1 µg/ml) in TE buffer for 30 min at 37°C. After post-fixation (4% paraformaldehyde in $1 \times$ PBS, 5 min, 4°C) and PBS washing, 2×5 min, the sections were acetylated in a 0.1 M triethanolamine (TEA) buffer (pH 8) with 0.25% acetic anhydride. TEA blocks endogenous activity of alkaline phosphatase, and acetanhydride reduces probe stickiness.

Incubation with 40 µl pre-hybridisation buffer at 37°C for 2 h preceded the pre-hybridisation, which was performed in a moist chamber. To prevent evaporation, the specimens were covered with parafilm. The probe cocktail (10 ng in 40 µl of hybridisation buffer) was added and left to hybridise overnight at 37°C, which is 23°C below T_m. This temperature was chosen because of short oligonucleotides. The slides were then washed in $2 \times$ SSC (2 $\times 5$ min), $1 \times$ SSC (2×15 min) and 0.25 SSC (2 $\times$ 15 min) in a shaking water bath at 37°C. The slides were then immersed in buffer I (100 mM Tris-HCl, 150 mM NaCl; pH 7.5) for 2×10 min, followed by treatment with buffer II (buffer I + 0.5% blocking reagent), to block non-specific anti-DIG binding. The
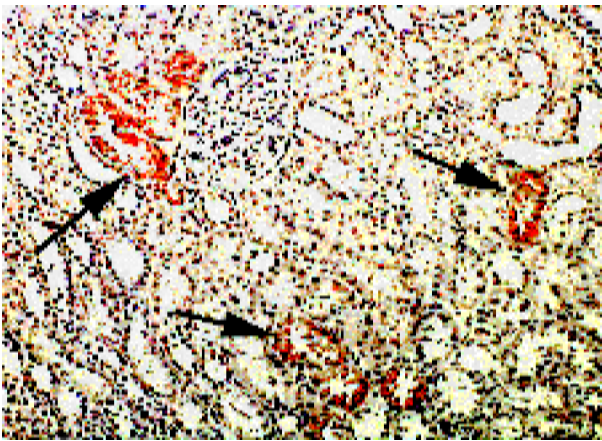
FIG. 1. Immunohistochemical staining for SLPI on healthy kidney biopsies, with goat anti-SLPI diluted 1/1000 and DAB as a chromogen, showing positive signal in the tubuli. Scale bar, 20 μm.
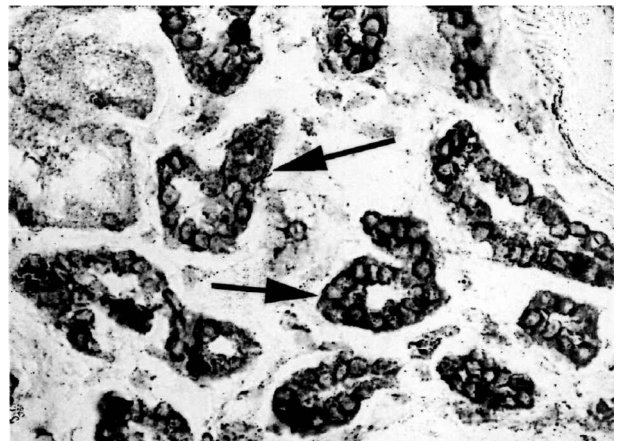

FIG. 2. Demonstration of SLPI mRNA by *in situ* hybridisation of healthy kidney biopsies. The specimens were hybridised using an equimolar cocktail of three 30-base long antisense, DIG-labelled oligonucleotides as probes. Positive staining is seen in the tubuli. Scale bar, 80 μm.

sections were then placed in a moist chamber and the anti-DIG-AP conjugate (1/500 in buffer I, 0.1% Triton X-100 and 1% normal sheep serum) was applied. Repetition of buffer I washing was carried out for 2×10 min. The slides were then placed in buffer III (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$; pH 9.5) for 10 min. Visualisation was achieved by incubation in 10 ml buffer III, 45 μl of NBT, 35 μl of BCIP and 1 mM Levamisole (240 mg/ml) for 2–4 h. The sections were then mounted using an aqueous mounting solution. Control sections were incubated with the matched sense-probes or with hybridisation buffer without probes.

Results

Immunohistochemical staining for SLPI was carried out on healthy kidney biopsies with positive staining results (Fig. 1) in the distal tubuli. In negative control sections incubated with adsorbed anti-SLPI antiserum, the positive staining was completely abolished. As seen in Fig. 2, a positive hybridisation signal was obtained in the tubuli cells of the healthy kidney biopsies. In negative controls, incubated without probes and with sense probes, respectively, no positive signal was seen.

Discussion

SLPI is considered to be a major protease inhibitor in the respiratory as well as in the gastrointestinal and genital tracts. A potential role in the urinary tract could therefore be anticipated. In this study, both immunohistochemistry and *in situ* hybridisation (ISH) showed clearly positive results, with no background staining and adequate controls. The positive staining of SLPI in distal tubuli in immunohistochem-

istry could be explained by renal filtration, but ISH also show a positive signal for SLPI mRNA in tubuli. These results imply local SLPI production in normal kidney, which is something that has not been demonstrated before. Our primary antibody has been used in many previous studies with clear-cut results, and must be considered highly specific.[12] For our ISH, we used an oligonucleotide cocktail synthesised for us by R&D Systems. These probes have worked well in previous studies, and the negative results obtained with the matched sense-strand probes confirm the specificity of the reaction for SLPI.[12] The known function of SLPI is local inhibition of activity and release of proteolytic enzymes.[1,10] Considering this, the role of SLPI in kidney would be to take part in a local regulation of proteolytic activity in various inflammatory conditions. SLPI might also, considering its antimicrobial and antiviral effects,[1,2] be of importance in the defence against ascending urinary tract infections. Of course, one could also anticipate new mechanisms for SLPI action in this context. Hence, the conclusion of this paper is that we have been able to demonstrate a novel local SLPI production in kidney, the role of which needs to be further elucidated.

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