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Production of secretory leucocyte protease inhibitor (SLPI) in human pancreatic β-cells

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Secretory leucocyte protease inhibitor (SLPI) is a potent inhibitor of granulocyte elastase and cathepsin G, and also an inhibitor of pancreatic enzymes like trypsin, chymotrypsin and pancreatic elastase. SLPI has also been shown to inhibit HIV-1 infections by blocking viral DNA synthesis. Since SLPI is an inhibitor of pancreatic proteases we wished to investigate whether SLPI was also actually produced in the pancreas. M-RNA from human pancreatic tissue showed evidence of SLPI production using the reverse transcriptase polymer chain reaction technique (RT-PCR). Using immunohistochemical methods SLPI was demonstrated in the β-ce1ls of the islets of Langerhans. The function could be local protease/antiprotease regulation or antiviral/antibacterial defence in the close vicinity of the cell surface, or even inside the β-cell itself.

Key words: SLPI, Islets of Langerhans, Pancreas, β-Cell, Antiviral, Antibacterial

Introduction

SLPI is an acid-resistant 11.8-kDa non-glycosylated serine antiprotease. Originally isolated from human parotid gland secretions,[1] it is found in cells on a variety of mucosal surfaces. For example, the seminal vesicles, prostate, epididymis and cervix,[2–7] in serous cells of the parotid and submandibular salivary glands,[1,8,9] in the upper respiratory tract, in the lacrimal glands,[10] as well as in the Clara cells and goblet cells of the lower respiratory tract.[11–13] SLPI has also been found in von Ebner's glands at the base of the tongue and submucosal cells of the oesophagus.[10] Recently, SLPI was found in Paneth cells in the small intestine, and in scattered mucosa cells of goblet-type in the epithelium of both the large and small bowel. In addition, SLPI has frequently been found in colonic adenomas.[14]

SLPI is thought to be an inflammatory regulator with strong inhibitory capacity aimed towards proteases present in various body fluids. Thus, SLPI is a potent inhibitor of leucocyte elastase and cathepsin G, and a moderate inhibitor of trypsin and chymotrypsin.[1,15,16] Furthermore, it is a moderate inhibitor of pancreatic elastase,[17] and it is also known to inhibit both mast cell chymase activity and histamine release from mast cells.[18,19] Its proteinase-inhibitory activity is known to be located in the second COOH-terminal domain. Although no inhibitory capacity has been detected in the NH2-terminal domain, antimicrobial capacity has been reported.[20,21] It has also been reported that SLPI blocks the human immunodeficiency virus type 1 by blocking DNA synthesis.[22] SLPI is probably involved in inflammatory regulation in the intestines as well as in the respiratory and urogenital tracts. In patients with ulcerative colitis, active pancreatic proteases have been found in faeces. Since SLPI is an inhibitor of both pancreatic and leukocytic proteases, it could be of importance in the pathogenesis of ulcerative colitis. Having recently demonstrated SLPI production in the intestinal mucosa,[14] and knowing that SLPI produced in the upper respiratory tract is rapidly degraded in gastric and duodenal juice,[23] we also wanted to investigate other possibilities of origin. Earlier studies failed to show production or presence of SLPI in the pancreas.[10] The aim of this study was, therefore, to reinvestigate the possible presence and production of SLPI in pancreatic tissue using more sensitive methods.

Methods and materials

Xylene, ethanol, hydrochloric acid, hydrogen peroxidase, formaldehyde, haematoxylin and Tris-buffered saline (0.05 mol/l Tris in NaCl 0.15 mol/l, pH 7.6) were standard chemicals for laboratory use. Recombinant human SLPI was a kind gift from Robert Thompson at Synergen Inc. (Boulder, CO, USA). Goat anti-SLPI antiserum was produced in the laboratory by immunising Swedish landrace goats with recombinant SLPI. Normal goat and rabbit serum was obtained from Dakopatt AB (Copenhagen, Denmark). Biotinylated rabbit anti-goat IgG antibodies and avidin-biotinylated horseradish peroxidase complex (ABC

complex) were purchased from Vector Laboratories (Burlingame, CA, USA). Diaminobenzidine was a product of Saveen Biotech AB (Malmö, Sweden). Crystalline3 porcine pepsin was obtained from Sigma Chemicals (St. Louis, MO, USA).

RT-PCR and Southern blot

Human pancreas Poly(A^+) RNA was obtained from Clontec (Palo Alto, CA, USA). Specific primers (R&D Systems Europe Ltd, Abingdon, UK) were designed according to the nucleic acid sequence of SLPI cDNA.[24] The upstream and downstream primers for amplification of the human SLPI cDNA fragments were: 5′-TGT CCT GAC ACT TGT GGC AT-3′ and 5′-CGA TCA ACT GGC ACT TCT TG-3′. The oligonucleotide used for probing the Southern blot of the RT-PCR product had the sequence 5′-GGT TTG GGG TGT CAA CAG GAT CCA GGC AT-3′ with the modification 3′+5′ digoxigenin.[24]

The first strand of cDNA was synthesised using reverse transcriptase, isolated from avian myeloblastosis virus (Appligene, Illkirch, France) in a 50-µl reaction mixture containing 2.5 µl 400 mM Tris–HCl, pH 8.3, 2.5 µl 400 mM KCl, 1 µl 300 mM $MgCl_2$, 5 µl 100 mM dithiothretiol (DTT), 5 µl 5 mM 4dNTPmix, 2 µl 2 mg/ml actinomycin D, 1 µl 4 µM downstream primer and approx. 1–2 µg poly(A^+) RNA (approx. 10 µl). The reaction mixture was incubated at 42°C for 60 mm, then diluted with 450 µl 10 mM Tris–HCl/10 mM EDTA, pH 7.5. To 5 µl of this diluted mixture were added 5 µl of each amplification primer, 20 mM each, 4 µl 5 mM 4dNTPmix, 10 µl 10× amplification buffer (500 mM KCl, 100 mM Tris–HCl, pH 8.4, and 1 mg/ml gelatine), 1.5 µl 100 mM $MgCl_2$ and 69 µl H_2O. The PCR reaction was performed using the 'hot start' method with paraffin pellets, heating at 94°C for 2 min followed by chilling on ice. Then 2.5 units (0.5 µl) of Taq DNA polymerase (Appligene) were added to the tubes. Amplification was carried out in a thermo-cycler (Hybaid Omnigene, Teddington, UK) with the following cycling programme: 39 cycles of 2 min at 55°C; 2 min at 72°C and 1 min at 94°C; followed by one cycle of 2 min at 55°C and 7 min at 72°C. The PCR product was then analysed by agarose gel electrophoresis and visualised after ethidium bromide staining (0.8% agarose gel in 0.5×TBE buffer).

The PCR-product was transferred to a Hybond N transfer membrane (Amersham, Buckinghamshire, UK) according to the method of Maniatis *et al.*[26] After heating the membrane for 30 min at 80°C, it was prehybridised for 4 h at 42°C in a solution containing 5× standard saline citrate (SSC), 50% formamide, 0.02% (w/v) sodium dodecylsulfate (SDS), 0.1% (w/v) *N*-lauroylsarcosine and 2% (w/v) blocking reagent (Boehringer-Mannheim, Mannheim, Germany). Hybridisation was carried out in the same solution, including 20 ng probe/ml solution overnight at 42°C.

After hybridisation the membrane was washed in 2 × SSC containing 0.1% SDS, 1 × 15 min, 0.1 × SSC containing 0.1% SDS, 2 × 15 min, both at room temperature. Detection of the target DNA hybrid was achieved as follows. After a brief rinse in 100 mM Tris–HCl, 150 mM NaCl, pH 7.5, the membrane was incubated in 0.5% (w/v) blocking reagent in the same buffer for 30 min. This was followed by a brief wash in buffer. Diluted anti-digoxigenin antibody–AP-conjugate (1:500) was applied to the membrane for 30 min. Rinses in buffer, 2 × 15 min followed. The staining was carried out by incubating the membrane in a solution containing 45 µl Nitroblue Tetrasodium salt (NBT), 35 µl 5-bromo-4-chloro-33 indolyl phosphate (X-phosphate) in 10 ml buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). The experiment was repeated three times.

Immunohistochemical staining

Pancreas specimens from five different patients collected during surgery for pancreatic adenocarcinoma were investigated. The specimens were from micro- and macroscopically normal pancreatic tissue. The patients were two men and three women between 41 and 69 years of age. They had no earlier history of pancreatic disease. Informed consent for the procedure was obtained. The tissue samples were cut at random and fixed in 4% formaldehyde. Paraffin-embedded tissue was routinely stained with haematoxylin for light microscopic examination. The fixed specimens were subjected to pepsin digestion (pepsin 4 mg/ml in 0.01 mol/l HCl) for 30 min at 37°C and were transferred into Tris-buffered saline. To quench endogenous tissue peroxidase activity, in the next step, the sections were incubated with 0.3% H_2O_2 in methanol for 30 min at room temperature. To block non-specific staining, the specimens were incubated for 10 min in 5% normal rabbit serum. In order to show specific staining in the β-cells, the specimens were treated as follows.

(1) SLPI

The slides were incubated with goat anti-human SLPI (1:1000) for 30 min followed by the addition of biotinylated rabbit anti-goat IG antibodies (5 µg/ml buffer) for 30 min. This was followed by incubation with avidin biotinylated horseradish peroxidase complex for 30 min. After the sections were rinsed, the substrate reaction was performed by incubating the sections in 3-amino-9-ethylcarbazole (AEC; Dako® Corporation, Carpinteria, CA, USA) for 25 min. After final washing the specimens were rinsed in tap water then mounted. All incubations were performed at room temperature, and specimens were washed three times (5 min) between each step with Tris-buffered saline. Negative controls were always incubated in parallel with adsorbed anti-SLPI antiserum which had

been produced by affinity chromatography on an SLPI-conjugated Sepharose 4B column (Pharmacia Fine Chemicals, Uppsala, Sweden). The experiments were repeated three times.

(2) Insulin

The procedure was identical with that described above, except that a sheep anti-human insulin antibody was used in 1:2000 solution ('The binding site', Birmingham, UK). Incubation was performed in alkaline phosphatase complex instead of avidin biotinylated horseradish peroxidase. For staining, 22.8 µl NBT and 17 µl BCIP were added to 5 ml buffer.

(3) Double staining

The specimens were first stained for insulin, then the same specimen was also stained for SLPI, as described above.

Results

RT-PCR and Southern blot analysis

Water was used as a negative control, and trachea tissue as a positive control. A characteristic band was observed in the trachea tissue lane representing SLPI cDNA fragments. In the lane representing pancreatic tissue there was specific staining of the corresponding amplified cDNA fragments in the same position, but somewhat weaker than for the positive tracheal control. The bands were distinct and the lanes contained no detectable signs of any other bands. There were no detectable bands in the lane containing the negative control (Fig. 1). The pattern was identical in all experiments.

Immunohistochemical staining

Immunoreactive SLPI was found using the human anti-SLPI antibody, resulting in bright red staining clearly concentrated to the islets of Langerhans (Fig. 2A). Using human anti-insulin antibodies, immunoreactive insulin was found in the β-ce1ls of the islets of Langerhans, as indicated by distinct blue staining (Fig. 2B). When the specimens were stained first with the human anti-insulin antibody, and then with the human anti-SLPI antibody, the original staining colours of blue and bright red were not observed, but the cells were stained purple resulting from insulin and SLPI in the same cell population (Fig. 2C). There was no sign of SLPI staining when using adsorbed anti-SLPI antiserum. The results obtained were verified in triplicate experiments.

Discussion

SLPI is found in many different cell types in man. Its generally accepted function has hitherto been protease inhibition, thereby modulating the inflammatory response. Protection of the ciliated epithelium of the respiratory tract against the activity of leucocyte proteases released in purulent infection is assumed to be one important function of SLPI.[15] New properties of this protein have recently been discovered, and there are now reports indicating antibacterial and antiviral effects.[20–22] In the light of these findings, new mechanisms for SLPI action may be anticipated. This study shows evidence of SLPI production in the β-cells of the islets of Langerhans. Utilising the RT-PCR technique we first observed SLPI-mRNA in pancreatic tissue. Using immunohistochemical methods we were then able to confirm that SLPI is produced in the β-cells of the islets of Langerhans. The function of SLPI in the islets of Langerlians is far from clear. The question arises of whether SLPI with pancreatic origin is meant to act extracellularly in the traditional protease inhibitor manner, or whether it has other functions. The anti HIV-l activity of SLPI has been shown to take place early in the viral infection of cells, probably after virus binding and before reverse transcription.[22] Our finding of SLPI in the pancreatic islets of Langerhans indicates an auto-/or paracrine function rather than an

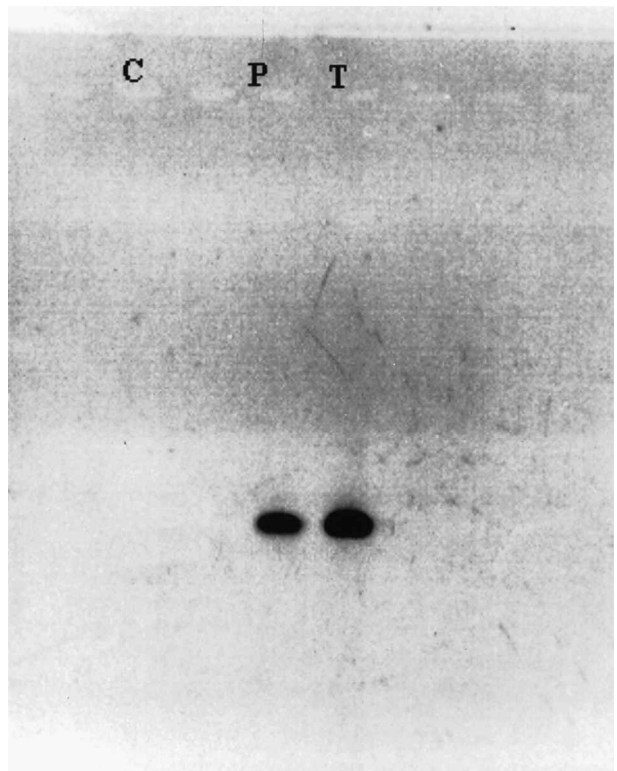

FIG. 1. SLPI cDNA fragments were demonstrated in pancreatic tissue using RT-PCR and Southern blot. C, negative control containing water; P, pancreatic tissue; T, tracheal tissue used as a positive control. In the lane representing pancreatic tissue strong specific staining of the corresponding amplified cDNA fragments was observed. The band is at the same position but somewhat weaker than that for the positive tracheal control. No bands were detected in the lane containing the negative control.

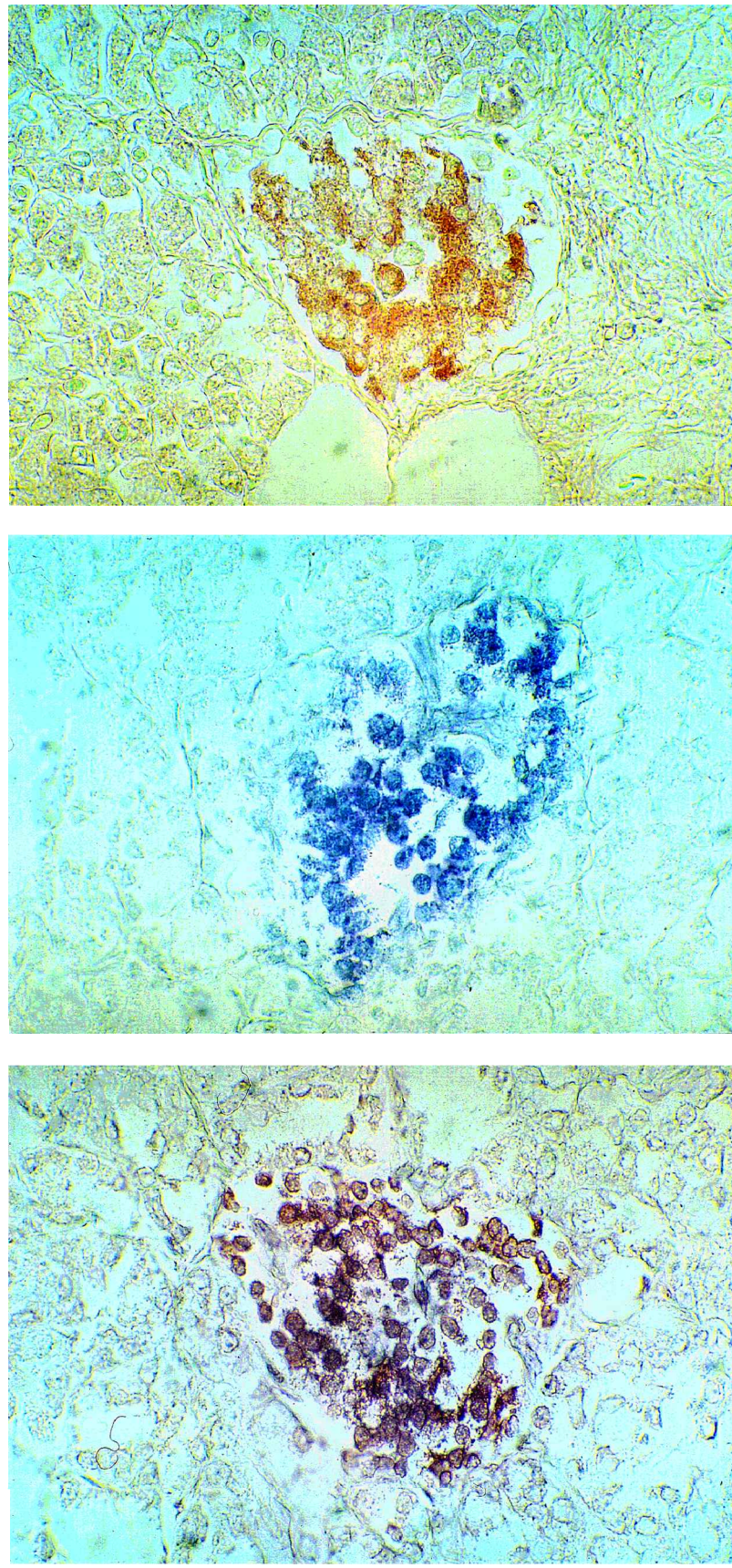

FIG. 2. Using immunohistochemical methods and staining with different antibody solutions novel colour schemes can be accomplished. (A) The presence of immunoreactive SLPI was demonstrated using a specific human anti-SLPI antibody resulting in a bright red colour in the islets of Langerhans. (B) Human anti-insulin antibodies showed blue staining in the β-cells of pancreas tissue. (C) Staining the specimens first for insulin then for SLPI resulted in a purple colour indicating the presence of insulin and SLPI in the β-cells of the islets of Langerhans.

exocrine function. In accordance with findings of anti HIV-1 activity and antibacterial properties, a possible function might be the protection of the cells against viral or bacterial infections. Another possible autocrine function could be protease/antiprotease regulation. Further studies are, however, required to identify the proteases involved.

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