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Expression of Inter-α-Trypsin Inhibitor Light Chain (Bikunin) in Human Pancreas[1]

Hiroshi Itoh, Masaki Tomita, Takahiko Kobayashi, Hirofumi Uchino, Haruhiko Maruyama, and Yukifumi Nawa[2]

Department of Parasitology, Miyazaki Medical College, 5200 Kihara, Kiyotake, Miyazaki 889-16

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Expression of inter-α-trypsin inhibitor light chain (ITI-LC, also known as bikunin or urinary trypsin inhibitor) was examined in various human tissues. By reverse-transcription polymerase chain reaction, the mRNA was detected not only in the liver, a known site of ITI-LC production, but also in the kidney, heart, lung, and pancreas. By RNA blot analysis, the mRNA was also detected in the pancreas and liver, but not in the kidney, heart, or lung. The ITI-LC protein was immunohistochemically detected along the surface of pancreatic acinar cells. These results indicate the apparent expression of the gene for ITI-LC in the pancreas. ITI-LC protein on the surface of pancreatic acinar cells may play an important role in preventing autodigestion by exocrine enzymes such as trypsinogen and chymotrypsinogen.

Key words: bikunin, human, inter-α-trypsin inhibitor light chain, pancreas, urinary trypsin inhibitor (UTI).

Inter-α-trypsin inhibitor (ITI) protein family (ITI, pre-α inhibitor, and inter-α-like inhibitor) consists of unique plasma proteinase inhibitors composed of combinations of one or two heavy chains (HCs) and/or one common light chain (LC) associated with chondroitin sulfate. The common LC (ITI-LC), also known as bikunin or urinary trypsin inhibitor (UTI), has two active Kunitz-type proteinase inhibitor domains (*1-4*) and the inhibitory activity of the ITI protein family entirely depends on ITI-LC. ITI-LC is co-produced with α1-microglobulin (α1mG), another plasma protein belonging to the lipocalin superfamily (*5*), by translation from the common *AMBP* gene followed by proteolytic processing (*6*). Liver has been thought to be their only source, because they were produced and secreted into conditioned media as an active form by human hepatoma HepG2 cells (*2, 4, 7, 8*) and rat primary cultured hepatocytes (*9, 10*). In addition, α1mG/ITI-LC cDNAs were cloned from livers of human and some other animal species, and the mRNAs were abundantly detected in this organ (*6, 11-14*). However, other possible production sites of the components of the ITI protein family have not been examined in detail. We examined the expression of human *AMBP* gene in various human tissues, and found that the pancreas could be an additional site of ITI-LC production.

MATERIALS AND METHODS

Tissues—Human tissues (skin, tongue, lung, liver, spleen, kidney, small intestine, pancreas, and heart) without apparent pathological changes taken from two autopsy cases (case 1: acute myocardial infarction; case 2: chronic myelocytic leukemia) were supplied by the 1st and 2nd Department of Pathology, Miyazaki Medical College. They were divided into two portions. One was used for extraction of total RNA by TRIZOL™ (Life Technologies, Gaithersburg, MD). The other was fixed in Zamboni solution (*15*) at 4°C overnight and then dehydrated in an ascending series of ethanol. After dehydration, the tissues were cleared in Lemosol (Wako Pure Chemical, Osaka) and embedded in paraffin wax for the following immuno-histochemical analysis.

RT-PCR Analysis—Five micrograms of total RNA was reverse-transcribed with random hexamer [pd(N)$_6$] and SuperScript reverse transcriptase (Life Technologies). The resulting cDNA was divided into two parts and each part was subjected to PCR for 30 cycles with the following primer sets: ITI-LC sense (5′-AAGAAAGAAGACTCCTG-CCAGCT-3′) and antisense (5′-TCACCAGGGACACCGC-AGTA-3′) or control β-actin sense (5′-GACTACCTCATG-AAGATCCT-3′) and antisense (5′-GTGGCCATCTCTTG-CTCGAA-3′). The cDNA sequences of human α1mG/ITI-LC and β-actin were taken from Refs. *6* and *16*, respectively. The thermal cycle profile was 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. The PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide.

RNA Blot Analysis—Twenty micrograms of total RNA was electrophoresed on 1% formaldehyde agarose gel and

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[2] To whom correspondence should be addressed. Phone: +81-985-85-0990, Fax: +81-985-84-3887, E-mail: paras@macqm.miyazaki-med.ac.jp

Abbreviations: ITI, inter-α-trypsin inhibitor; LC, light chain; HC, heavy chain; α1mG, α1-microglobulin; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; MCP, mast cell protease; PSTI, pancreatic secretory trypsin inhibitor; UTI, urinary trypsin inhibitor.

transblotted onto Hybond-N nylon membrane (Amersham, Buckinghamshire, UK). Hybridization was performed under a high stringency condition using ^{32}P-labeled probes as described (*17*). The specificity of the probe used [corresponding to the nucleotide positions 928-1097 of human α1mG/ITI-LC (*6*)] has been confirmed (*17*). The control human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe was purchased from Clontech (Palo Alto, CA). After hybridization, the membranes were washed with 0.1×standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at 65°C for 30 min×2 times and then exposed to Kodak XRP-5 films at −70°C for 3 days. Alternatively, the intensities of mRNA signals in pancreas and liver were directly compared by using a Bioimaging Analyzer, Fujix BAS 2000 system (Fuji Photo Film, Tokyo) (*18, 19*).

Immunohistochemistry—Antiserum against highly purified ITI-LC derived from normal human urine, which was kindly supplied by Mochida Pharmaceutical (Tokyo), was raised in a rabbit. Secondary antibody (peroxidase-labeled swine anti rabbit IgG) and peroxidase-anti-peroxidase (PAP) rabbit polyclonal antibody were purchased from DAKOPATTS (Glostrup, Denmark). Serial 4-μm-thick sections were deparaffinized and rehydrated before staining. For immunohistochemistry, the sections were immersed in methanol containing 0.6% H_2O_2 for 20 min at room temperature to block the endogenous peroxidase activity. After incubation with 3% bovine serum albumin for an hour at 37°C to block non-specific protein binding, the sections were incubated with the primary antiserum against human ITI-LC (1:500) at 4°C overnight. Subsequently, sections were incubated with the secondary antibody (1:500) for an hour and thereafter with PAP complexes for 30 min at room temperature. The sections were visualized with the DAB/Metal Concentration (10×) and Stable Peroxide Substrate Buffer (1×) system (Pierce, Rockford, IL). They were washed with water, and counterstained with hematoxylin.

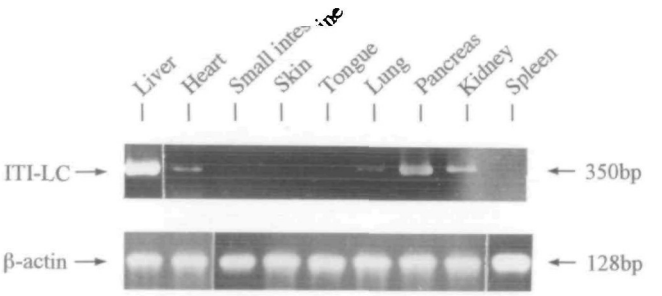

Fig. 1. **RT-PCR analysis of α1mG/ITI-LC in various human tissues.** The mRNA was detected not only in the liver, a known producing site of ITI-LC, but also in the kidney, heart, lung, and pancreas. β-Actin primers were used as a control of total RNAs applied.

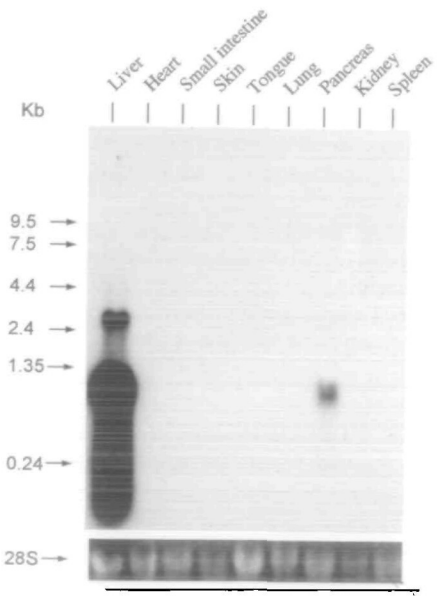

Fig. 2. **RNA blot analysis of α1mG/ITI-LC in various human tissues.** The mRNA was detected in the pancreas and liver, but not in other tissues. The 28S ribosomal RNA is shown as a control for the amount of total RNA.

RESULTS

By sensitive RT-PCR analysis (Fig. 1), α1mG/ITI-LC mRNA was detected in the liver, pancreas, kidney, heart, and lung, but not in other tissues. By RNA blot analysis (Fig. 2), the mRNA was detected only in the liver and pancreas with predominance in the liver at a size of 1.2 kb, but not in other tissues even after a long exposure (2 weeks). The α1mG/ITI-LC mRNA was consistently de-

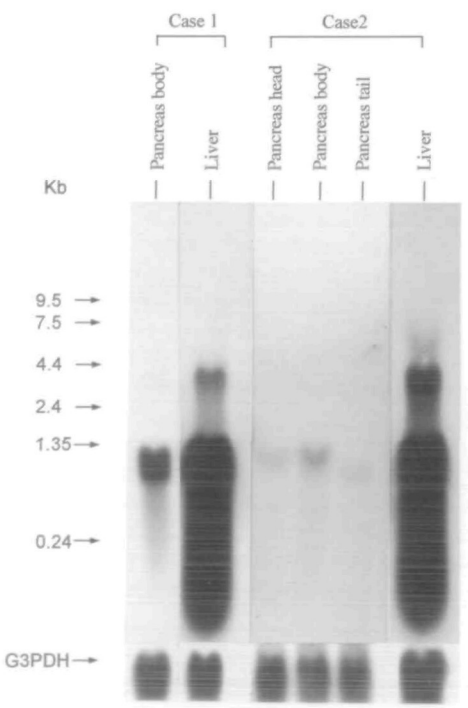

Fig. 3. **RNA blot analysis of α1mG/ITI-LC in human liver and pancreas of two autopsy cases.** In case 2, total RNAs were derived from three distinct portions of human pancreas. Note that the α1mG/ITI-LC mRNA was consistently detected in pancreatic tissues from two autopsy cases and the levels were comparable among three portions (head, body, and tail) of the pancreas. The amount of the mRNA in the pancreas was approximately 7% of that in liver based on simple quantification by a Bioimaging Analyzer BAS 2000 using G3PDH as a control.

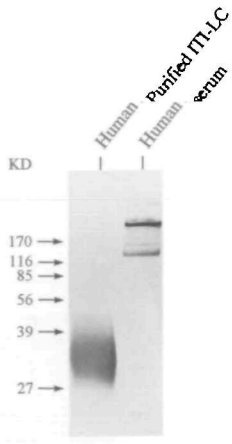

Fig. 4. **Specificity of the antiserum to human ITI-LC confirmed by Western blot analysis.** The antiserum used specifically recognized human purified ITI-LC and human serum ITI (225 kDa), pre-α inhibitor (125 kDa), and ITI-related derivatives (*2*).

tected in pancreatic tissues from two autopsy cases and the levels were comparable among three portions (head, body, and tail) of the pancreas (Fig. 3). The relative amount of the α1mG/ITI-LC mRNA with respect to G3PDH mRNA in the pancreas was approximately 7% of that in the liver from simple quantification by the Bioimaging Analyzer BAS 2000. These results indicate that the ITI-LC mRNA is *in fact* transcribed from the gene in human pancreas, as well as the liver.

To examine the distribution of ITI-LC protein in various human tissues, we developed rabbit polyclonal antibody against human purified ITI-LC protein. Specificity of the antibody was examined by Western blot analysis of the purified ITI-LC protein and normal human serum (Fig. 4). The results show that the antiserum used specifically recognized human purified ITI-LC and human serum ITI (225 kDa), pre-α inhibitor (125 kDa), and ITI-related derivatives (*2*). The broad band of the purified ITI-LC was thought to reflect a high degree of glycosylation of this peptide (*20*). By immunohistochemical staining with this antiserum (Fig. 5), positive reaction was observed along the surfaces of pancreatic acinar cells, while no reaction was seen in islet cells or in ductal cells. A positive reaction was also seen in the blood vessels, but not in pancreatic ducts. Pre-immunization sera from the same rabbit did not show positive staining and the positive staining with the primary antiserum was completely abolished after absorption with an excess amount of purified ITI-LC protein.

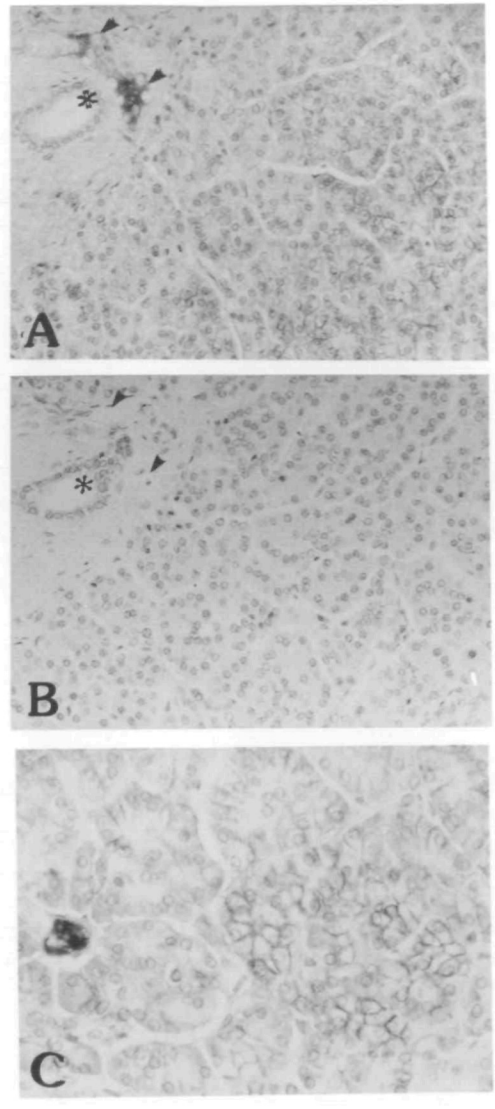

Fig. 5. **Immunohistochemistry of ITI-LC protein in human pancreas.** Positive staining with anti ITI-LC antiserum was observed along the surface of the pancreatic acinar cells. A positive reaction was also seen in the blood vessels (arrowhead) but not in pancreatic duct (*) (A). Positive staining was completely abolished after absorption with an excess amount of purified ITI-LC protein (B). High power view showing positive staining on acinar cell surfaces and no staining in islet cells or in ductal cells (C). (A: ×120, B: ×120, C: ×500; counterstained with hematoxylin)

DISCUSSION

ITI-LC has two active Kunitz-type proteinase inhibitor domains, is produced in liver and is present in plasma alone or in combination with one or two heavy chains, forming a unique plasma proteinase inhibitor family (*1-4*). The present results show that, in addition to liver, the pancreas also produces ITI-LC. Besides ITI-LC, two other proteinase inhibitors are known to be produced in the pancreas: one is pancreatic secretory trypsin inhibitor (PSTI) (*21*) and the other is basic pancreatic trypsin inhibitor (BPTI) (*22*). PSTI is a Kazal-type inhibitor and is abundantly expressed in pancreatic acinar cells and mainly secreted into pancreatic juice (*23, 24*). Although BPTI, which is also known as aprotinin, is a Kunitz-type inhibitor, it has only a single inhibitor domain and has been found only in bovine pancreas, but not in the pancreas of other animals, including humans (*25*). Thus, ours is the first report of the expression of a Kunitz-type proteinase inhibitor in human pancreas.

ITI-LC protein and its derivatives were also found in gastrointestinal tract, kidney, brain, and liver as well as in some tumor cells and tissues, plasma, urine (UTI), lymph, and bile (*20, 26-29*), mainly by immunohistochemical analysis. However, in the present study, α1mG/ITI-LC mRNAs were detected in the liver and pancreas, but not in other tissues (except for the brain and hepatoma cells) by RNA blot analysis, although the mRNA was detected in the

kidney by sensitive RT-PCR analysis. The discrepancy between the immunohistochemical and RNA blot analyses in this tissue might be due to uptake of ITI-LC from the serum. Concerning this matter, we recently reported that rat mast cell proteinase inhibitor, trypstatin, is a fragment of ITI-LC, and α1mG/ITI-LC mRNA was not detected in mast cells even by RT-PCR analysis, suggesting that ITI-LC protein is taken up into various cells, including mast cells, from the plasma and lymph (*17*).

In the present study, the amount of ITI-LC mRNA detected in pancreas was far lower than that in liver. Thus, though the size of the organs must also be taken into account, ITI-LC produced in pancreas may make little contribution to the plasma level of ITI-LC. Since immunohistochemical study revealed that ITI-LC protein was present on the surface of the pancreatic acinar cells but not in the pancreatic ducts, it may play a role in local regulation of exocrine enzymes and/or protection of acinar cells themselves from secreted and activated enzymes by linking with its specific receptor on the surfaces (*30*). In contrast to ITI-LC, PSTI is secreted into pancreatic juice (*23, 24*). Furthermore, ITI-LC can inhibit both trypsin and chymotrypsin (*1*), whereas PSTI can inhibit trypsin but not chymotrypsin (*23*). Therefore, the physiological roles and functions of ITI-LC in human pancreas may be different from those of PSTI. Conversely, since both ITI-LC and PSTI have a growth-promoting effect on endothelial cells (*7*), they may act synergistically as acute phase reactants during various pathological conditions, such as pancreatitis and malignant tumor (*31, 32*). In connection with this, ITI-LC protein, named urinastatin (Miraclid™), has been used for the treatment of acute pancreatitis. In this respect, the present results will increase our understanding of the pathophysiological function of the pancreas and facilitate further investigation on the biological roles of ITI-LC protein.

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