Pancreatic Stone Protein of Pancreatic Calculi in Chronic Calcified Pancreatitis in Man

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ABSTRACT

Context The role of protein components of pancreatic secretions has been controversial in pancreatic stone formation.

Objective To study the lithogenic role of pancreatic stone protein and lactoferrin in stone formation in chronic pancreatitis.

Patients Pancreatic stones were collected from 13 patients with alcoholic (n=6) and nonalcoholic (n=7) chronic calcified pancreatitis.

Main outcome measures Pancreatic stone extracts were analyzed for pancreatic stone protein and lactoferrin using enzyme immunoassay. The localization of pancreatic stone protein immunoreactivity in the stone was observed using immunogold staining and scanning electron microscopy.

Results Immunoreactivities for pancreatic stone protein were detected in the stones from all 13 patients with chronic calcified pancreatitis and for lactoferrin in the stones from five of the 13 patients. Pancreatic stone protein immunoreactivity distributed diffusely from the center to the periphery of the pancreatic stones.

Conclusions Involvement of pancreatic stone protein seems to be constant from the initial step of the stone formation to subsequent steps of the stone growth. However, pancreatic stone protein is only one of the precipitating proteins in pancreatic secretions such as lactoferrin, trypsinogen, etc.

INTRODUCTION

Stone formation in the pancreatic duct system is common in chronic pancreatitis. However, the mechanism of the stone formation has not been fully elucidated. In a canine experimental model of pancreatolithiasis, persistent stasis of protein-rich pancreatic juice secondary to partial obstruction in the pancreatic duct leads to the calculus formation [1, 2]. Plugs formed by the precipitation of the protein within the interlobular and intralobular ducts are one of the earliest findings in chronic pancreatitis and the protein plugs subsequently perpetuate inflammation of the gland through repeated obstruction of the pancreatic duct system. If concentration-dependent precipitation is a cause of the protein plug formation, some proteins must be increased, at least, in their concentration. Through the analysis of pancreatic juice, the iron-binding protein, lactoferrin, has been found to be secreted in greater amounts by patients with chronic pancreatitis [3, 4, 5, 6, 7]. Lactoferrin may play a role in the formation of the protein plugs frequently seen in chronic pancreatitis because of its ability to produce an aggregation of a large acidophilic protein, such as albumin [8].
Pancreatic stone protein (PSP) is a 16 kDa acidic protein with an isoelectric point in the range of pH 5.5-6. A truncated form of this protein was originally isolated from calcium carbonate stones surgically removed from the main pancreatic duct of humans with chronic pancreatitis [9]. PSP was believed to serve as an inhibitor of calcium carbonate precipitation in pancreatic juice and was called “lithostathine” by some [10]. Increasing numbers of studies suggest that PSP has no more crystal inhibitory activity than endogenous proteins such as serum albumin and trypsinogen [11, 12]. PSP is highly susceptible to trypsin cleavage at its ARG11-ILE12 bond [13].

The addition of bovine trypsin to human pancreatic juice enhances the conversion of soluble isoforms of PSP S2-5 into an insoluble isoform of PSP-S1 [14]. The presence of a small amount of free proteolytic activity has been reported in the pure pancreatic juice obtained from patients with pancreatitis [15, 16, 17, 18]. Localization of the trypsin immunostaining in the center of the pancreatic stones was confirmed in our previous report [19]. When the intraductal activation of trypsinogen to trypsin occurs, soluble PSP S2-5 is converted into insoluble PSP S1 with the subsequent precipitation of PSP S1.

In the present report, we studied the lithogenic role of PSP and lactoferrin by measuring the content of the proteins in pancreatic stones using enzyme immunoassay, immunostaining of PSP, and scanning electron microscopy.

METHODS

Collection of Pancreatic Stones

A total of 423 patients with chronic pancreatitis (alcoholic calcified 124, alcoholic non-calcified 135, non-alcoholic calcified 57, non-alcoholic non-calcified 107) were followed up in Nagoya University Hospital and its affiliated hospitals during the period 1970-1991. Pancreatic stones were collected from 13 patients (12 males, 1 female; mean age 51.0±15.4 years): 6 patients had alcoholic (A: daily intake of more than 80 g of ethanol over 10 years) chronic calcified pancreatitis at surgery or autopsy (one patient) and 7 patients had non-alcoholic (NA) chronic calcified pancreatitis at surgery as listed in Table 1.

Body mass index and serum albumin exceeded 18.5 kg/m² and 3.0 g/dL, respectively, in all 13 patients without clinical evidence of malnutrition, although the average diet compositions were unknown in

<table>
<thead>
<tr>
<th>Patient (Sex, age)</th>
<th>Stone size</th>
<th>Weight (mg)</th>
<th>Protein (µg/mg stone)</th>
<th>Pancreatic stone protein (ng/µg protein)</th>
<th>Lactoferrin (ng/µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alcoholic pancreatitis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1 (male, 71)</td>
<td>Large</td>
<td>244.2</td>
<td>3.6</td>
<td>10.4</td>
<td>0.1</td>
</tr>
<tr>
<td>A2 (male, 52)</td>
<td>Small</td>
<td>16.0</td>
<td>28.8</td>
<td>419.2</td>
<td>1.0</td>
</tr>
<tr>
<td>A3 (male, 51)</td>
<td>Small</td>
<td>66.7</td>
<td>4.2</td>
<td>31.3</td>
<td>0</td>
</tr>
<tr>
<td>A4 (male, 42)</td>
<td>Large</td>
<td>73.3</td>
<td>6.1</td>
<td>105.0</td>
<td>0</td>
</tr>
<tr>
<td>A5 (male, 41)</td>
<td>Small</td>
<td>8.5</td>
<td>87.0</td>
<td>0.1</td>
<td>3.0</td>
</tr>
<tr>
<td>A6 (male, 34)</td>
<td>Small</td>
<td>61.7</td>
<td>6.9</td>
<td>291.5</td>
<td>0</td>
</tr>
<tr>
<td><strong>Nonalcoholic pancreatitis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA1 (male, 74)</td>
<td>Large</td>
<td>26.2</td>
<td>7.6</td>
<td>1.7</td>
<td>0</td>
</tr>
<tr>
<td>NA2 (male, 70)</td>
<td>Small</td>
<td>16.4</td>
<td>13.7</td>
<td>1.3</td>
<td>0</td>
</tr>
<tr>
<td>NA3 (female, 69)</td>
<td>Large</td>
<td>54.6</td>
<td>5.9</td>
<td>18.9</td>
<td>7.3</td>
</tr>
<tr>
<td>NA4 (male, 50)</td>
<td>Small</td>
<td>46.4</td>
<td>7.8</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>NA5 (male, 46)</td>
<td>Large</td>
<td>317.7</td>
<td>8.9</td>
<td>92.1</td>
<td>2.5</td>
</tr>
<tr>
<td>NA6 (male, 35)</td>
<td>Large</td>
<td>197.0</td>
<td>11.4</td>
<td>225.1</td>
<td>0</td>
</tr>
<tr>
<td>NA7 (male, 29)</td>
<td>Large</td>
<td>92.7</td>
<td>6.7</td>
<td>19.1</td>
<td>0</td>
</tr>
</tbody>
</table>

*Stone size: large stone indicates maximal diameter of stone greater than 5 mm; small stone means all stones smaller than or equal to 5 mm in diameter.*
detail. The stones were well-rinsed in saline to remove contaminating superficial proteins, then dried in air, and maintained in this condition until they were prepared for this study.

**Extraction of Proteins from Stones**

The stones were ground in a mortar in 3 ml of 0.5M ethylenediaminetetracetic acid (EDTA), pH 8.0, and kept overnight at 4 ºC after a 30-min sonication. The next morning, the suspension was ground again, then dialyzed using two liters of distilled water three times in semipermeable tubing with a cutoff of 3.5 kDa and centrifuged for 20 min at 18,000 rpm.

**Measurement of Protein, Pancreatic Stone Protein and Lactoferrin**

The supernatant of the pancreatic stone suspension was analyzed for protein by Lowry’s method [20], for human pancreatic stone protein immunoreactivity [14, 21] and lactoferrin [6, 7] by enzyme immunoassay methods, as previously reported.

**Molecular Forms of Pancreatic Stone Protein in Pancreatic Stone**

The supernatant of the stone suspension was applied to a Mono S (HR 5/5, Pharmacia. Uppsala, Sweden) column equilibrated with 0.02 M sodium acetate buffer (pH 4.0) containing 0.02 M CaCl₂. The column was washed with the buffer for 5 min at a flow rate of 1.0 mL/min, then eluted for 35 min with a linear gradient of 0-1.0 M NaCl as previously reported [14, 21, 22]. Enzyme immunoassay of PSP S₂-5 and PSP S₁ in each fraction was carried out using the method described previously [14, 21, 22].

**Localization of Human Pancreatic Stone Protein Immunoreactivity in Pancreatic Stones**

Localization of pancreatic stone protein immunoreactivity in pancreatic stones was studied using immunogold staining and scanning electron microscopy as previously reported [19]. Pancreatic stones were embedded in Epon. The epoxy blocks were polished using a grinding machine (EMP-2, Eiko Engineering. Nakaminato, Japan) at 280 rpm with a polish powder of aluminum oxide (diameter 0.05 µm) in order to expose the inner part of the stones. The exposed surface of the pancreatic stone was immunostained using the first antibody (mouse anti-human pancreatic stone protein, the same as used in the enzyme immunoassay for human pancreatic stone protein) and colloidal gold (20 nm gold protein) conjugated goat anti-mouse 1gG (EY Laboratories Inc. San Mateo, CA, USA). After ion-sputter-coating with gold-palladium, the immunostained surface was observed with a scanning electron microscope (S-800, Hitachi. Tokyo, Japan) at 5 kV as previously reported [19].

**ETHICS**

Informed consent was obtained from each patient and the study protocol was approved by the Human Research Ethics Committee of the Nagoya University School of Medicine.

**STATISTICS**

Data are presented as mean and standard deviation. ANOVA was applied for statistical comparison. The correlation coefficient was calculated by the least-squares method. P values less than 0.05 were considered statistically significant.

**RESULTS**

**Protein, Pancreatic Stone Protein and Lactoferrin Content in Pancreatic Stones**

Protein, human pancreatic stone protein immunoreactivity and lactoferrin immunoreactivity in pancreatic stones were determined and presented as µg/mg of stone for protein and ng/µg of protein for pancreatic stone protein and lactoferrin immunoreactivities (Table 1). The protein
content was not significantly (P=0.285) different between alcoholic (22.8±32.9 µg/mg stone) and non-alcoholic (8.9±2.8 µg/mg stone) pancreatitis.

The pancreatic stone protein immunoreactivity was measured in pancreatic stones in all 13 patients, ranging from 0.1 to 419.2 ng/µg protein, but did not differ significantly (P=0.238) between alcoholic (142.9±173.4 ng/µg protein) and non-alcoholic (51.2±83.2 ng/µg protein) pancreatitis.

There was a significant correlation (P=0.010) between protein and pancreatic stone protein in pancreatic stones when the alcoholic pancreatitis patient (A5) was excluded because the pancreatic stone of the patient was predominantly composed of fatty acid calcium [19] (Figure 1). No further significant correlation was detectable between pancreatic stone protein and lactoferrin (P=0.894). The lactoferrin immunoreactivity was detectable in 5 of the 13 patients, ranging from 0.1 to 7.3 ng/µg protein. There was no definite common etiology or clinical feature to explain the high lactoferrin content in patients A5, NA3, and NA5. The stone in the A5 patient was predominantly composed of fatty acid calcium. The stones in the NA3 and NA5 patients were large and were obtained from the markedly dilated main pancreatic duct.

Molecular Forms of Pancreatic Stone Protein in Pancreatic Stones

To identify the molecular forms of pancreatic stone protein immunoreactivity in pancreatic stones, the pancreatic stone extracts from two patients (A1 and NA6) were applied to the Mono S column. The elution peak of the pancreatic stone protein immunoreactivity was not identical to that of pancreatic stone protein S2,5 or S1 previously reported [14, 23] (Figure 2).

Localization of Pancreatic Stone Protein in Pancreatic Stones

Localization of the pancreatic stone protein immunoreactivity in pancreatic stones was observed by immunogold staining and scanning electron microscopy (Figures 3 and 4). The exposed inner surface of the pancreatic stone from patient NA6 looked amorphous at the center of the stone with concentric laminar layers partly in the periphery (Figure 3). Gold particles were distributed diffusely from the center to the periphery of the pancreatic stone.

DISCUSSION

Analysis of the protein components in the pancreatic study is important in clarifying the mechanism of stone formation in the pancreatic duct. In the present study we have shown that pancreatic stone protein is a major
protein component of the pancreatic stone. Pancreatic stone protein was detectable in pancreatic stones from all the 13 patients studied, ranging widely from only a trace amount to 1.21%, when expressed as a percentage of the stone weight. Mariani et al. [24] reported that the smallest amount was 0.013% and the largest was 0.296% of the stone weight. The percentages obtained in our study showed wider variation than that of Mariani et al. [24]. In the present study, a significant correlation was obtained between total protein and pancreatic stone protein when patient A5 was excluded because the pancreatic stone was composed predominantly of fatty acid calcium and not of calcium carbonate. A wide range of percentages of pancreatic stone protein in the total protein (ranging from 0.01 to 41.9 %) suggests that the mechanisms and protein components involved in the stone formation are multifactorial, and that pancreatic stone protein is not the sole protein involved in stone formation [3, 4, 5, 6, 7, 13, 15, 19, 25]. In our previous study, trypsin immunoreactivity was detectable in pancreatic stones in 11 of the 13 patients with chronic pancreatitis [18]. The stones without detectable trypsin immunoreactivity were from two of the three patients having a high content of lactoferrin in the stones. Of the two patients, one (A5) had a pancreatic stone predominantly composed of fatty acid calcium and the other (NA5) had large stones migrating in the markedly dilated pancreatic duct. In these patients, recurrent infection with or without persistent stasis in the pancreatic secretion might be involved in the stone formation. Reports of bacterial growth in stone debris [26] and impaired antibacterial activity in pure pancreatic juice from patients with chronic pancreatitis [27] support the involvement of the infection in the stone formation. However, pancreatic stone protein contents were very low in A5 but moderately high in NA5. The inconsistency in protein constituents might reflect the complexity of mechanisms and precipitable proteins involved in the stone formation.

Tympner demonstrated increases of viscosity, trypsin activity, lactoferrin and total protein in pure pancreatic juice obtained from patients with chronic pancreatitis [28]. Renner et al. also observed high concentrations of protein through the period of secretin stimulation and the sporadic appearance of free proteolytic activity in many 1-min specimens through the collection period of pure pancreatic secretions in patients with acute pancreatitis. A small amount of precipitate in several 1-min collections of pancreatic secretions appeared to precede and coincide with the presence of...
free proteolytic activity [16, 17]. In our previous studies, both activation of the pancreatic juice with enteropeptidase and the addition of trypsin to the pancreatic juice converted the soluble forms of pancreatic stone protein S_{2.5} into the insoluble form of pancreatic stone protein S_1 and about 45-85% precipitated out after 1 h [14, 22]. In a study by Provansal-Cheylan et al., analysis of the clogging material in occluded pancreatic endoprosthesis revealed the presence of trypsinogen, amylase and pancreatic stone protein S_1 [29].

The localization of the pancreatic stone protein immunoreactivity in the pancreatic stone was demonstrated by immunostaining using an immunogold technique and scanning electron microscopy. Gold particles, indicating the presence of pancreatic stone protein immunoreactivity, distributed diffusely from the center to the periphery of the pancreatic stones. In our previous study, trypsinogen immunoreactivity was distributed more densely in the center of the pancreatic stone than the periphery of the stone [19]. From the density of the distribution of gold particles, the involvement of trypsinogen in the initial step of the stone formation seems to be more intense than in the subsequent steps of stone growth. On the other hand, involvement of pancreatic stone protein seems to be constant from the initial step to the subsequent steps of stone growth. In physiological conditions, pancreatic stone protein may play an inhibitory role in calcium carbonate precipitation as one of several components in pancreatic juice which normally prevents calcium carbonate precipitation. In pathological conditions where local activation of pancreatic zymogen occurs [16, 17, 18, 25], pancreatic stone protein plays a lithogenic role by transforming the soluble forms of pancreatic stone protein S_{2.5} into the insoluble form of S_1 rather than playing an inhibitory role in stone formation.

We cannot conclude which protein is more important for the precipitate and stone formation. The most likely candidate for promoting precipitation would be the local activation of pancreatic zymogens. Active trypsin would further enhance precipitation by cleavage of soluble pancreatic stone protein S_{2.5} to insoluble protein S_1. However, pancreatic stones containing only trace amounts of pancreatic stone protein and trypsinogen suggest that additional candidate proteins and mechanisms will be required in stone formation.

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Key words Calculi; Lactoferrin; Pancreatic Ducts; Pancreatitis; Proteins

Abbreviations PSP: pancreatic stone protein

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