PANCREATIC BIOPSY AS A PROCEDURE FOR DETECTING IN SITU AUTOIMMUNE PHENOMENA IN TYPE 1 DIABETES

Close Correlation Between Serological Markers and Histological Evidence of Cellular Autoimmunity

Akihisa Imagawa,1 Toshiaki Hanafusa,3 Shinji Tamura,1 Makoto Moriwaki,1 Naoto Itoh,4 Koji Yamamoto,2 Hiromi Iwahashi,1 Kazuya Yamagata,1 Masako Waguri,2 Takao Nanmo,1 Sae Uno,1 Hiromu Nakajima,1 Mitsuyoshi Namba,6 Sumio Kawata,1 Jun-ichiro Miyagawa,1 and Yuji Matsuzawa1

To better understand the pathogenesis of type 1 diabetes, we have developed pancreatic biopsy under laparoscope for recent-onset type 1 diabetic patients. The patients included 29 acute-onset type 1 diabetic patients, 5 latent-onset type 1 diabetic patients, and 1 type 2 diabetic patient. Their median age was 28 years, and the duration of diabetes at the time of biopsy was \( \sim 3 \) months. In 31 of 35 patients, we could obtain the pancreas tissue by punching. No serious complications, such as heavy bleeding, peritonitis, or pancreatitis, have been experienced. Pneumoderma was observed in two patients, and abdominal dull pain had continued for 2 days in two patients. However, special treatment was not necessary for these complications. T-cell–predominant infiltration to islets (insulitis) and hyperexpression of major histocompatibility complex class I antigens on islet cells were the two major findings and were observed in 17 of 20 recent-onset type 1 diabetic patients. These findings could be regarded as evidence of immune attack against \( \beta \)-cells, and their presence was closely correlated with the presence of either anti-GAD or anti–IA-2 antibodies \((P = 0.02)\). In conclusion, pancreatic biopsy under laparoscope is a safe procedure without serious complications, according to our findings, for detecting in situ autoimmune phenomenon in recent-onset type 1 diabetic patients. Diabetes 50: 1269–1273, 2001

SINCE BOTTAZZO ET AL. (1) REPORTED ISLET CELL ANTIBODIES (ICAs) IN THE SERA OF TYPE 1 DIABETIC PATIENTS, AN ACCUMULATING AMOUNT OF EVIDENCE HAS SUGGESTED THAT TYPE 1 DIABETES MAINLY RESULTS FROM AUTOIMMUNE PANCREATIC \( \beta \)-CELL DESTRUCTION (2–4). SEVERAL ISLET AUTOANTIGENS, SUCH AS INSULIN, GAD, PROTEIN TYROSINE PHOSPHATASE, ISLET ANTIGEN (IA)-2, AND IA-2B, HAVE BEEN IDENTIFIED (4–5). HOWEVER, ANTIBODIES TO THESE AUTOANTIGENS DO NOT APPEAR TO PLAY A PATHOGENIC ROLE, BUT T-CELLS WOULD PLAY CRUCIAL ROLES IN DESTROYING THE PANCREATIC \( \beta \)-CELLS IN THE DEVELOPMENT OF TYPE 1 DIABETES (3). SEVERAL LABORATORIES HAVE TRIED TO DETECT CYTOTOXIC T-CELLS FROM THE PERIPHERAL BLOOD OF TYPE 1 DIABETIC PATIENTS (6), BUT CONCLUSIVE RESULTS HAVE YET TO BE ESTABLISHED.

WE HAVE TAKEN ANOTHER APPROACH TO DETECT THE EVIDENCE OF PANCREATEIC \( \beta \)-CELL DESTRUCTION BY AUTOACTIVE T-CELLS (7–8). IN SITU CHARACTERIZATION OF VARIOUS PHENOMENA OCCURRING IN THE ISLETS OF TYPE 1 DIABETIC PATIENTS WOULD PROVIDE MORE DIRECT EVIDENCE THAN ANY OTHER TESTS AVAILABLE FOR PATHOGENIC MECHANISMS. PANCREAS BIOPSY UNDER THE LAPAROSCOPE HAS BEEN REPORTED TO DIAGNOSE PANCREATITIS, PANCREATIC TUMOR, AND OTHER Pancreatic diseases (9–10). WE HAVE APPLIED THIS METHOD TO RECENT-ONSET TYPE 1 DIABETIC PATIENTS AND HAVE ESTABLISHED A METHOD FOR HISTOLOGICAL EXAMINATION OF PANCREATEIC BIOPSY SPECIMENS.

RESEARCH DESIGN AND METHODS

PATIENTS. Pancreatic biopsies were performed in 35 patients (Table 1). All patients were ketosis-prone and fulfilled the criteria of the American Diabetes Association for type 1 diabetes (4,11). Of the 35 patients, 29 (21 males) had acute-onset type 1 diabetes, 5 (3 males) had latent-onset type 1 diabetes (12), and only 1 male had type 2 diabetes with ketoacidosis at onset (13). Their median age was 28 years and ranged from 16 to 50. All patients were thin, and their median BMI was 18.6 kg/m² and ranged between 14.2 and 21.6. The median duration from the time of diagnosis with type 1 diabetes to the time of biopsy was 3 months (0–13).

This study was approved by the Ethics Committee of the Osaka University Medical School. Patients were fully informed of the purpose of biopsy (to elucidate the pathogenesis of \( \beta \)-cell death) and that the study would have no immediate benefit to the patient, but might be of help when effective immunotherapy is developed. After that, written informed consent was obtained from all patients.

Patients had been treated with intensive insulin therapy after the onset of overt diabetes. Their plasma glucose levels were well controlled at the time of biopsy.
Pancreatic biopsy. The patients were fasted at least 12 h before biopsy. Their plasma glucose levels were controlled by intravenous insulin infusion before and during the biopsy. As a sedative, 15 mg pentazocine (Pentagin; Sankyo, Tokyo) and 25 mg hydroxyzine (Atarax-P; Pfizer, Tokyo) were given intramuscularly 30 min before the biopsy. Laparoscopy was carried out by a standard technique using a laparoscope (Olympus, Tokyo) under local anesthesia with lidocaine hydrochloride (Xylocaine; Astra Japan, Tokyo). First we administered 1.5–2.0 l of air into the abdominal cavity and maintained it throughout the procedure. Next, the laparoscope was inserted from two fingerbreadths below the umbilicus. Then, the head end of the operating table was raised to about 30°, and the lesser omentum under the left lobe of the liver was exposed as much as possible. The left hepatic lobe was then elevated by a sound (bougie), which was inserted from approximately two fingerbreadths below xiphoid process. The puncture site of the sound was finally determined by inspection through the laparoscope. When there were few intra-abdominal fat deposits, which was usually the case in type 1 diabetic patients at diagnosis, the body of the pancreas could be observed through the transparent lesser omentum, between the lower edge of the left hepatic lobe and the lesser curvature of the stomach.

After inspection of the pancreas, a biopsy forceps (Olympus) was inserted into the peritoneal cavity from approximately two fingerbreadths to the left of the site where the sound was inserted. The puncture site of the forceps was finally determined by inspection through the laparoscope. After the fatty part of lesser omentum was moved aside by the forceps, we punched the body of the pancreas through the thin membrane of the lesser omentum. We paid special attention not to injure any visible vessels. Matchhead-sized specimens were taken once or twice. After the biopsy, we carefully examined the punched site of the pancreas for at least 10 min until we were certain of no further bleeding. The biopsy was then completed after confirmation of hemostasis through inspection of the peritoneal cavity as well as the biopsy site. Protease inhibitor (gabexate mesilate, FOY; One Pharmaceutical, Tokyo) and antibiotics (cefazolin sodium hydrate, Cefamezin; Fujisawa Pharmaceutical, Osaka, Japan) were prophylactically infused for 24 h after the biopsy. Total blood count, serum amylase, and lipase levels were measured every 12 h in addition to plasma glucose levels before and after the biopsy. Part of the methods of pancreatic biopsy was reported previously (7–8).

Immunohistochemistry. Obtained biopsy tissues were immediately frozen in dry ice acetone and isopentane, and 5 μm-thick sections were cut on a cryostat. Approximately 300 consecutive sections were made from each biopsy tissue, stored at −80°C until assay, and used for immunohistochemical study as reported previously (7,8).

We used double-immunofluorescent method to detect insulitis, mononuclear cell infiltration to the islet, and expression of major histocompatibility complex (MHC) antigens. Sections were air-dried for 30 min, prefixed in cold acetone for 10 min, washed three times in phosphate-buffered saline (PBS) (pH 7.4), and incubated overnight at 4°C with the following monoclonal antibodies: mouse anti-human T-cell CD3 (57B4B5; Dakopatts, Glostrup, Denmark), B-cell (L26; Dakopatts), macrophage (EBM11; Dakopatts), MHC class I antigens (W6/32; Serotec, Oxford, U.K.), and MHC class II antigens (84H10; Immunotech, Marseille, France). After washing in PBS, the sections were incubated for 60 min at room temperature with biotinylated horse anti-mouse immunoglobulins (Vector Laboratories, Burlingame, CA) and then for an additional 30 min with fluorescein isothiocyanate–conjugated avidin (Vector Laboratories). After the first staining, the sections were incubated with guinea pig anti-insulin (Dakopatts) or rabbit anti-glucagon antibodies (kindly provided from Dr. Iwasa, Takeda Chemical, Osaka, Japan) and followed by the corresponding secondary antibody, rhodamine-conjugated rabbit anti–guinea pig (Zymed Laboratories, South San Francisco, CA) or Texas red–conjugated donkey anti–rabbit (Amersham International, Amersham, U.K.) immunoglobulins.

When two or more mononuclear cells (CD3+ T-cells, macrophages, or B-cells) were shown in an islet, we diagnosed it as insulitis-positive. Because one mononuclear cell was sometimes seen in normal islets, it was difficult to judge such findings as pathologic or incidental (8). To establish this protocol, we examined control tissues obtained from normal parts of the pancreas body of five subjects who underwent partial pancreatectomy for gastric cancer. The glucose tolerance of these subjects was within normal range before the operation.

Ilet autoantibodies. The following autoantibodies were examined at the time of biopsy. ICAs were determined by an indirect immunofluorescent method, and the cutoff value was 5 Juvenile Diabetes Foundation units (JDF U). Anti-GAD antibodies were measured by a radioimmunoassay kit (Rip-GAD; Hoechst Japan, Tokyo; or GAD-Ab “Cosmic”; Cosmic Corporation, Tokyo). A value >5 U/ml using the former kit and 1.5 U/ml using the latter kit was considered positive. Anti–IA-2 antibodies were measured with an immunoprecipitation assay kit (Cosmic Corporation), and the cutoff value was 0.75 U/ml (14).

Statistical analysis. Statistical analysis was performed using Fisher’s exact probability test.

RESULTS
Outcome of pancreatic biopsies. Pancreatic tissue was available in 31 of 35 patients (Table 2). The punctured sites are shown in Fig. 1. In these patients, the body of the pancreas was observed through the transparent lesser omentum, between the lower edge of the left hepatic lobe and the lesser curvature of the stomach as shown in Fig. 2. One to three specimens were obtained in these patients. The size of each specimen was ~2–6 mm long, and the weight was 20–40 mg. Pancreatic tissue was not available in the other four patients (Table 2). The laparoscope could not be inserted because of excess tension of abdominal muscle in one patient. The pancreas was not visible due to fat deposits in one 50-year-old male patient whose BMI was 21.6 kg/m². The amount of pancreatic tissue was too small for histological study in the two other patients. Obtained tissue was almost occupied by fat in these patients. Their BMI was 18.0 and 19.7 kg/m², respectively.

Complications of pancreatic biopsies. During the pancreatic biopsy, the patients’ plasma glucose was well controlled. As shown in Table 3, we had no serious
complications, such as bleeding that necessitated surgical treatment, leakage of pancreatic juice, peritonitis, and pancreatitis. The estimated blood loss was ~1 ml. Pneumoderma was observed in two patients, and abdominal dull pain had continued for 2 days after the biopsy in two other patients. However, special treatment was not necessary for these minor complications.

Leukocytosis (white blood cell count 11,000/mm$^3$) was observed in one patient 1 h after the biopsy, but the patient recovered 12 h later without any special treatment. Hyperamylasemia was observed in two other patients, and the maximum level of serum total amylase was 141 and 249 IU/l, respectively (normal range 40–120). Both patients did not reveal leukocytosis and abdominal pain; therefore, these patients were not diagnosed with pancreatitis. In 28 other patients whose pancreatic tissue was obtained successfully and in 4 patients whose pancreatic tissue was not obtained, no leukocytosis, hyperamylasemia, and hyperlipasemia was observed.

**Immunohistochemistry.** Pancreatic biopsy specimens were analyzed immunohistochemically in 31 patients with available pancreatic tissue. All 30 type 1 diabetic patients were found to have markedly decreased insulin-containing β-cells, which was the case for type 1 diabetes, but also to harbor residual β-cells. In a type 2 diabetic patient with ketoacidosis at onset, β-cells were well preserved.

In 29 recent-onset type 1 diabetic patients, we examined (mean ± SD) 77 ± 75 sections containing at least one islet on average. As a result, 15 patients revealed insulitis and 2 patients showed hyperexpression of MHC class I antigens in islets without insulitis. In those biopsy specimens demonstrating insulitis, and by using an islet counting method, 28.3 ± 21.0% of the islets were determined to be affected islets. Hyperexpression of MHC class I antigens in islets was observed not only in β-cells but also in α-cells (Fig. 3). Insulitis and hyperexpression of MHC class I antigens in islets were two major immunological abnormal findings shown in 17 patients, whereas only 1 patient had ectopic MHC class II antigen expression in islet cells (data not shown). This patient also showed insulitis and hyperexpression of MHC class I antigens in islets. The presence of insulitis or hyperexpression of MHC class I antigens in islets were closely correlated, as shown in Table 4. The remaining 12 patients revealed neither insulitis nor abnormal expression of MHC antigens in islets (Fig. 3); however, 3 of 12 patients revealed T-cell–predominant infiltration to exocrine pancreas (data not shown). In 13 of 15 insulitis-positive patients, infiltrating cells were CD3$^+$ T-cell–predominant, whereas macrophage-predominant insulitis was observed in 2 other patients, and B-cell–predominant insulitis was not observed in any patient. The detailed analysis of infiltrating cells in a part of our patients were reported previously (8).

In two patients without recent-onset type 1 diabetes, neither insulitis nor hyperexpression of MHC class I antigens in islets were observed; one patient was diag-

![FIG. 2. Laparoscopic picture showing a pancreatic body of a type 1 diabetic patient. P, pancreas; L, liver.](image)

![FIG. 3. Photomicrographs of pancreatic biopsy specimens. CD3$^+$ T-cells (shown in green) are infiltrating into the islet (insulin-containing pancreatic β-cells are shown in red) in one patient (a), but are not observed in another patient (b). Expression of MHC class I antigens increased in one patient (c), but did not increase in another patient (d). Original magnification: ×280 (a, b, and d) and ×200 (c).](image)

<table>
<thead>
<tr>
<th>Complications of pancreatic biopsy</th>
<th>0 of 31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major</td>
<td></td>
</tr>
<tr>
<td>Bleeding (necessitating surgical treatment)</td>
<td>0</td>
</tr>
<tr>
<td>Leakage of pancreatic juice</td>
<td>0</td>
</tr>
<tr>
<td>Peritonitis</td>
<td>0</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>0</td>
</tr>
<tr>
<td>Minor</td>
<td></td>
</tr>
<tr>
<td>Pneumoderma</td>
<td>2</td>
</tr>
<tr>
<td>Abdominal dull pain 2 days after</td>
<td>2</td>
</tr>
</tbody>
</table>

**TABLE 3**

<table>
<thead>
<tr>
<th>Insulitis and hyperexpression of MHC class I antigens in islets in 29 recent-onset type 1 diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperexpression of MHC class I antigens</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>Insulitis-positive</td>
</tr>
<tr>
<td>Insulitis-negative</td>
</tr>
</tbody>
</table>

Data are $n$. $P = 0.003$.
nosed with type 2 diabetes, and the other’s biopsy was performed 13 months after the onset of overt type 1 diabetes.

**Islet autoantibodies.** In 29 recent-onset type 1 diabetic patients with available pancreatic tissue, ICAs were positive in 18 patients (62.1%), anti-GAD antibodies were positive in 14 patients (50.0% [not tested in 1 patient]), and anti-IA-2 antibodies were positive in 13 patients (44.8%). The presence of either anti-GAD or anti–IA-2 antibodies was closely correlated with that of in situ immunological abnormalities (insulitis or hyperexpression of MHC class I antigens in islets or both) \( (P = 0.02) \), as shown in Table 5. The presence of high-titer ICAs \( (>20 \text{ JDF U}) \) was also closely correlated \( (P = 0.01) \). Sensitivity and specificity of these islet autoantibodies and the combination of them are shown in Table 6.

**DISCUSSION**

First, we have demonstrated that laparoscopic pancreatic biopsy was a safe procedure in recent-onset type 1 diabetic patients. We have shown no serious complications in all 35 patients on whom a biopsy was performed. Only pneumoderma and abdominal dull pain, which needed no further treatment, were observed in two patients, respectively. Ishida (10) also reported that only 7 of 215 (3.3%) pancreatic biopsies revealed any complication: 2 patients had leakage of pancreatic juice, 3 experienced hemorrhage, 1 showed spillage of necrotic tissue into the peritoneal cavity, and 1 had acute pancreatitis. The diagnosis of their patients included acute or chronic pancreatitis and pancreas tumor. Vascular proliferation or dilatation of small pancreatic ducts was observed in pancreatic cancer and pancreatitis patients (15), but not in type 1 diabetic patients. Therefore, pancreatic biopsy under laparoscopy would be safer when applied to type 1 diabetic patients. This study could also pave the way for applying pancreatic biopsy to prediabetic individuals in a prevention trial of type 1 diabetes.

Second, we have demonstrated that recent-onset type 1 diabetic patients could be divided with regard to pancreatic histology; in particular, we are referring to the patients with insulitis and/or hyperexpression of MHC class I antigens in islets or the patients without either of them. The former patients could be classified into autoimmune type 1 (type 1A) diabetes; the latter could be idiopathic (type 1B) (4,16). To avoid the variability of insulitis (17), we confirmed that all 31 patients were found to harbor residual β-cells. We always took biopsy samples from the same position of the pancreas to decrease variation among the patients, and we examined sections from several different parts of the biopsy tissue \( (77 \pm 75 \text{ sections contained at least one islet on average}) \). We have shown that future β-cell function could be predictable from the analysis of biopsy specimens (14,18). Thus, it would be beneficial to diagnose with the histological subtype of type 1 diabetes at the onset of disease.

Third, pancreatic biopsy has enabled us to clarify the relation between the islet autoantibody status and the simultaneous histological findings in recent-onset type 1 diabetic patients. The presence of either anti-GAD or anti–IA-2 antibodies closely correlated with in situ immunological abnormalities in islets, which were considered to be evidence of cellular autoimmunity. The combination assay of anti-GAD antibodies and anti–IA-2 antibodies showed the highest sensitivity, although ICAs were still sensitive as a single serological marker. It has been reported that anti-GAD antibodies complement anti–IA-2 antibodies in type 1 diabetic patients (19). The prevalence of anti–IA-2 antibodies has been relatively low in adult-onset type 1 diabetic patients, which was also shown in the present study, but it complements anti-GAD antibodies even in adult-onset patients. Our study has shown the efficacy of measuring both anti-GAD and anti–IA-2 antibodies to predict immunologically abnormal histology in the islets.

Additionally, the analysis of pancreatic biopsy specimens gives us useful information on the pathogenesis of type 1 diabetes. We have reported that CD8\(^+\) T-cells were predominant in insulitis lesions, and there was a close relationship between insulitis and overexpression of MHC class I antigens in islet cells (8). These findings suggested that CD8\(^+\) T-cells play a crucial role in the pathogenesis of autoimmune type 1 diabetes via the recognition of autoantigens in association with hyperexpressed MHC class I molecules. In insulitis-positive patients, we have reported the involvement of apoptosis through the Fas-Fas ligand system in insulitis lesions (20). Several cytokines, such as tumor necrosis factor-α and γ-interferon, were also involved in the pancreas of type 1 diabetic patients (21). The CD28-B7 system would affect the activation of T-cells in insulitis lesions (22).

In conclusion, pancreatic biopsy under laparoscope is a safe procedure without serious complications, according
to our findings, for detecting in situ autoimmune phenomenon in recent-onset type 1 diabetic patients.

ACKNOWLEDGMENTS
This study was supported by grants from the Japanese Ministry of Education, Culture, and Science and the Japanese Ministry of Health and Welfare.

REFERENCES