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Expression of pancreatic digestive enzymes in normal and pathologic epithelial cells of the human gastrointestinal system

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Abstract Pancreatic digestive enzymes have rarely been reported in human nonpancreatic organs. We examined their expression in the epithelial cells of the nonpancreatic gastrointestinal organs, looking for pancreatic α-amylase, trypsin, chymotrypsin and pancreatic lipase. Western blotting, enzyme assay and pancreatic α-amylase mRNA were also used in selected specimens. In normal tissues, immunoreactivity of one or more of these enzymes was frequently noted in cells of the salivary glands, stomach, duodenum, large pancreatic ducts, extrahepatic bile ducts and gall bladder. The epithelium of the normal oesophagus, small intestine and colon were consistently negative for these enzymes. In pathologic tissues, immunoreactivity for one or more enzymes was present in epithelial cells of pleomorphic adenomas of the salivary glands, oesophageal squamous cell carcinoma, gastric adenoma and adenocarcinoma, pancreatic adenocarcinoma, cholecystitis, adenocarcinoma of the gall bladder and extrahepatic bile duct, and colon adenoma and adenocarcinoma. Western blotting showed a specific band of each enzyme in some specimens of normal stomach. In situ hybridization for pancreatic α-amylase mRNA showed specific signals in the normal stomach, but not in the normal colon. Reverse transcriptase polymerase chain reaction analysis for pancreatic α-amylase mRNA revealed specific signals in the normal stomach. Enzyme assay revealed that the stomach and gall bladder showed these activities. The data suggest that pancreatic digestive enzymes are produced by several epithelial cell types of the nonpancreatic gastrointestinal organs, that the organs positive for pancreatic enzyme have a common cell lineage, and that neoplasms continue to express or neoexpress these enzymes after neoplastic transformation.

Key words Pancreatic digestive enzymes · Immunohistochemistry · In situ hybridization · RT-PCR · Enzyme assay

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Abbreviations mRNA messenger ribonucleic acid, IgG immunoglobulin G, DNA deoxyribonucleic acid, dUTP deoxyuridine triphophate, dATP deoxyadenine triphosphate, dGTP deoxyguanine triphosphate, dTTP deoxythymidine triphosphate, dCTP deoxycytosine triphosphate, EDTA ethylenediaminetetraacetic acid, RT-PCR reverse transcriptase polymerase chain reaction, PBS phosphate-buffered saline, PFA paraformaldehyde, DIG digoxigenin, TE Tris-HCl-EDTA, SSC sodium chloride–sodium citrate solution, TNE Tris-HCl-NaCl-EDTA, kDa kilodalton, bp base pair

Introduction

Ingested foods are digested by enzymes located in the epithelial cells of the digestive organs. The pancreas is the major site of such digestive enzymes; it produces and secretes enzymes that digest proteins, peptides, saccharides, lipids and nucleotides [23]. These include pancreatic α-amylase (EC 3.2.1.1.), trypsin (EC 3.4.2.1.4.), chymotrypsin (EC 3.4.2.1.1.) and pancreatic lipase (EC 3.1.1.3.) [23]. The enzymes have been considered to be present exclusively in the pancreas [37], although lipase activity (nonpancreatic lipase) is present in several organs including the liver, adipose tissue and stomach [16, 17, 25, 34].

Recently, however, it has been revealed that intrahepatic large bile ducts, septal bile ducts and peribiliary glands express pancreatic α-amylase, trypsin and pancreatic lipase under normal conditions and in various hepatobiliary diseases [26–30, 32]. Hepatocytes are negative for the three pancreatic enzymes [26–29, 32]. These observations suggest that these pancreatic digestive enzymes are present in the epithelial cells of gastrointestinal organs other than the pancreas and intrahepatic bile

ducts. We therefore investigated the expression of four pancreatic digestive enzymes (pancreatic α-amylase, trypsin, chymotrypsin, and pancreatic lipase) immunohistochemically in various normal and pathologic epithelial cells of human gastrointestinal organs other than the liver and intrahepatic bile ducts. We also studied the enzymes proteins by the Western blot technique in selected specimens of normal stomach. Moreover, we evaluated the pancreatic α-amylase mRNA expression in selected specimens by in situ hybridization and reverse transcriptase polymerase chain reaction (RT-PCR) techniques, and examined the enzymatic activity or concentration in selected specimens by biochemical methods. With regard to terminology, although the term "expression" is generally used for the expression of mRNA, in this study the term "expression" was used for the expression of immunoreactive pancreatic digestive enzymes' proteins when not otherwise specified.

Materials and methods

We obtained normal (86 cases; age range 25–72 years; mean age 62 years; male 48, female 38) and pathologic tissues (103 cases; age range, 36–77 years; mean age 65 years; male 54, female 49) from the human gastrointestinal organs (other than the liver) from the recent surgical files (1993–1994) at our laboratory (Tables 1, 2). Material obtained at surgery was immediately fixed in 10% neutral formalin, and embedded in paraffin. Several 3-μm sections were obtained from each paraffin block, and one was stained with haematoxylin and eosin. The rest were subjected to immunohistochemical staining.

Table 1 Immunoreactive pancreatic digestive enzymes in normal epithelial cells of the gastrointestinal tract

Epithelial cells	*n*	Number (%) of cases positive for pancreatic digestive enzymes			
		Pancreatic α-amylase	Trypsin	Chymotrypsin	Lipase
Salivary glands	5				
Duct cells		0 (0%)	5 (100%)	0 (0%)	0 (0%)
Acinar cells		0 (0%)	0 (0%)	0 (0%)	0 (0%)
Oesophagus	10	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Stomach	16	7 (44%)	11 (69%)	12 (75%)	13 (81%)
Duodenum	8	3 (38%)	7 (88%)	5 (63%)	8 (100%)
Pancreas	5				
Large duct cells		5 (100%)	5 (100%)	5 (100%)	5 (100%)
Acinar cells		5 (100%)	5 (100%)	5 (100%)	5 (100%)
Gall bladder	12	4 (33%)	7 (58%)	5 (42%)	8 (67%)
Extrahepatic bile duct	5	2 (40%)	5 (100%)	5 (100%)	4 (80%)
Small intestine	12	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Colon	13	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table 2 Immunoreactive pancreatic digestive enzymes in pathologic epithelial cells of the gastrointestinal tract

Epithelial cells	*n*	Number (%) of cases positive for pancreatic digestive enzymes			
		Pancreas α-amylase	Trypsin	Chymotrypsin	Lipase
Salivary glands					
Sialoadenitis	3	0 (0%)	3 (100%)	0 (0%)	0 (0%)
Pleomorphic adenoma	5	0 (0%)	5 (100%)	0 (0%)	0 (0%)
Acinic cell tumour	1	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Oesophagus					
Squamous cell carcinoma	6	0 (0%)	1 (17%)	0 (0%)	0 (0%)
Stomach					
Adenoma	10	1 (10%)	4 (40%)	2 (20%)	5 (50%)
Adenocarcinoma	11	2 (18%)	4 (36%)	3 (27%)	6 (55%)
Pancreas					
Pancreatitis	4	4 (100%)	4 (100%)	4 (100%)	4 (100%)
Adenocarcinoma	8	3 (38%)	7 (88%)	3 (38%)	4 (50%)
Gall bladder					
Cholecystitis	17	5 (29%)	12 (71%)	8 (47%)	10 (59%)
Adenoma	2	0 (0%)	0 (0%)	1 (50%)	1 (50%)
Adenocarcinoma	7	2 (29%)	6 (86%)	3 (43%)	3 (43%)
Extrahepatic bile duct					
Adenocarcinoma	3	1 (33%)	3 (100%)	2 (67%)	2 (67%)
Colon					
Adenoma	15	0 (0%)	7 (47%)	0 (0%)	1 (7%)
Adenocarcinoma	10	0 (0%)	7 (70%)	0 (0%)	6 (60%)
Carcinoid tumour	1	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Fresh specimens of the normal stomach (*n*=5), normal colon (*n*=5) and normal pancreas (*n*=5) were obtained at surgery, snap frozen, and embedded in OTC compound. Several 6-μm frozen sections were briefly fixed with acetone, followed by the immunohistochemical examination for the pancreatic digestive enzymes.

Four paraffin or frozen sections from each case were stained immunohistochemically for pancreatic α-amylase, trypsin, chymotrypsin and pancreatic lipase, using the avidin-biotin-peroxidase complex method of Hsu et al. [10]. In brief, after deparaffinization and elimination of endogenous peroxidase activity, the sections were treated with normal serum for 20 min. Then, the sections were incubated at 4° C overnight with the monoclonal antibodies to human pancreatic α-amylase (mouse IgG1 class, dilution=1:320; approximate IgG concentration=7 μg/ml; Chemicon Inc., Tomecula, USA), the monoclonal antibody to human trypsin (mouse IgG1 class, dilution=1:400; IgG concentration=5 μg/ml; Chemicon), the monoclonal antibody to human chymotrypsin (mouse IgG1 class, dilution=1:400, IgG concentration=5 μg/ml, Chemicon), and the monoclonal antibody to human pancreatic lipase (mouse IgG1 class, dilution=1:500, IgG concentration=4 μm/ml, Chemicon). These monoclonal antibodies are IgG obtained from ascites derived from hybridoma lines according to the manufacturer's instruction. The antipancreatic α-amylase antibody does not react with salivary α-amylase [26]. The dilution or concentration of each antibody solution was determined using the control pancreatic specimens. That is, variously diluted antibody solutions were applied and the dilution showing the best results with no background staining was determined. The sections were then treated for one hour with biotinylated anti-mouse IgG (Vector Lab, Burlingame, Calif.) followed by treatment with the avidin-biotin-peroxidase complex (Vectastain ABC Kit, Vector Lab) for 1 h. Reaction products were developed by 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.03% hydrogen peroxide. Nuclei were lightly counterstained with haematoxylin.

Since the immunoreactivity was homogeneously negative or heterogenously positive, the immunohistochemical results were subjectively semiquantitated and classified into negative (–), and +, ++, and +++, in which the percentage of positive cells was 1–33%, 34–66%, and 67%–100%, respectively.

Specificity of immunostaining was tested by several methods. First, adult pancreas (*n*=5) was used as a positive control in each immunohistochemical run. Secondly, nonimmune mouse serum or phosphate-buffered saline (PBS) was substituted for the primary antibody solution, followed by the immunostaining. Thirdly, an absorption test was performed in each immunostaining. Each antibody solution was mixed with human pancreatic α-amylase (Athens Inc., Athens, Ga.), trypsin (Athens), chymotrypsin (Athens) or pancreatic lipase (Elastin Products, Owensville, Mich.). The mixed solutions were incubated at 4° C overnight with agitation, followed by centrifugation at 6,000 rpm for 10 min. The supernatant was collected and used as the primary antibody in each immunostaining. Fourthly, five irrelevant mouse monoclonal antibodies (MB-1 and MT-1, Bio-Science, Switzerland; anti-type IV collagen, Shiseido, Japan; anti-actin, Amersham, UK; MIB-1, Immunotech, France) which were developed in ascites and were at the same immunoglobulin concentration as the antibodies against the pancreatic enzymes, were used as primary antibodies, followed by the immunostaining. Finally, Western blot analysis was performed using normal pancreas (*n*=3) obtained from autopsy, as described later.

For Western blot analysis, normal pancreas (*n*=3) was obtained from autopsy, and the normal part of gastric mucosa was obtained from surgically resected stomachs (*n*=6). They were immediately snap-frozen and stored at –80° C until analysis. Proteins were extracted from these specimens by homogenization with lysis buffer and centrifugation. Western blot procedure was performed as previously described [33]. Detection of signals was performed by enhanced chemiluminescent technique using ECL kit (Amersham, Bucks., UK). Protein loads of 100 μg or 1500 μg were applied to the analysis. The antibodies employed were the same as those used in immunohistochemistry.

In situ hybridization for pancreatic α-amylase mRNA was performed on fresh normal stomach (*n*=5), pancreas (*n*=5) and colon (*n*=5), all obtained at surgery for cancer; serial frozen sections were made. They were fixed in 4% paraformaldehyde (PFA) in PBS for 15 min, incubated for 15 min at 37° C with 2.5 g/ml proteinase K in 10 mM Tris-HCl and 2 mM $CaCl_2$ and treated with 0.2 N HCl for 10 min. After postfixation for 10 min in 4% PFA in PBS, the slides were acetylated with 0.25% acetic anhydrate in 0.1 M triethanol amine for 10 min. The slides were dehydrated in graded ethanol and air-dried.

Three oligonucleotide antisense probes against exons 1, 5, and 6 of pancreatic α-amylase DNA [9] were synthesized by DNA automatic synthesizer (exon 1,5′-TGTTGTGTATTTGGGGAATACTGAGCCCAGCAGAACCC-3′; exon 5, 5′-CCGAGTTTTGCACCATACTTGAATTCTGTCACCCGGCC-3′; exon 6, 5′-TGTCAGAAGGTACGAAACCCCAACCTTCTCCCCAGTTC-3′). These probes were mixed and used for in situ hybridization. Oligonucleotide sense probes were also synthesized, and they were also mixed and used as negative controls. These oligonucleotides were labelled with the digoxigenin (DIG) Oligonucleotides Tailing Kit (Boehringer Mannheim Biochemica, Indianapolis, Ind.). Briefly, 100 pmol oligonucleotides, tailing buffer, $CoCl_2$ solution, DIG–dUTP solution, dATP solution and terminal transferase were mixed in a microfuge tube and incubated at 37° C for 60 min. The DIG-tailed oligonucleotides were precipitated by LiCl and ice-cold ethanol for 2 h at –80° C, and then spun by centrifugation at 12,000 *g* for 10 min at 4° C. The probes were further washed with 70% ethanol, dried under vacuum, and dissolved in 50 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA).

The slides were prehybridized for 1 h at 37° C with hybridization solution containing 50% deionized formamide, 10 mM Tris-HCl (pH 7.6), 200 μg/ml yeast tRNA, 1×Denhardt's solution, 10% dextran sulfate and 250 μg/ml salmon sperm DNA. Then the slides were incubated with hybridization solution containing 100 ng/ml DIG-tailed oligonucleotide probes in moist chamber at 37° C for 16 h. The sections were washed in 2×SSC containing 50% formamide for 30 min at 37° C, then in TNE buffer (10 mM Tris–HCl, 500 mM NaCl, 1 mM EDTA) for 10 min at 37° C, and twice in 0.2×SSC for 20 min at 37° C.

The slides were washed with DIG buffer 1 (100 mM Tris-HCl pH 7.6, 150 mM NaCl) briefly, blocked with 1.5% blocking reagents in DIG buffer 1 for 1 h, and further incubated in 500× diluted anti-DIG polyclonal antibody conjugated with alkaline phosphatase (Boeringer Mannheim Biochemica) for 2 h. The slides were washed twice in DIG buffer 1 for 15 min each, equilibrated with DIG buffer 3 (100 mM Tris-HCl pH 8.0, 100 mM NaCl, 50 mM $MgCl_2$) for 3 min, and incubated with colour substrate solution containing nitroblue tetrazolium salt (175 μg/ml) and levamizole (1 mM) in DIG buffer 3. The colour reaction was developed for 6–10 h in a darkroom, and stopped in DIG buffer 4 (10 mM Tris-HCl, 1 mM EDTA).

To carry out RT-PCR analysis, total cellular RNA was extracted from10 μm fresh-frozen sections from two cases of normal gastric tissue positive for immunoreactive pancreatic α-amylase by the guanidine thiocynate/phenol method using Isogen (Nippon Gene, Toyama, Japan), and 10 μg of total cellular RNA was converted into complementary DNA by reverse transcriptase (Strategene, La Jolla, Calif.).

Four oligonucleotide primers of pancreatic α-amylase DNA were made by DNA automatic synthesizer, with reference to exons of pancreatic α-amylase DNA sequence [9]. The sequence of the four primers was as follows: primer 1,5′-AAGTTCTTTCTGTTGCTTTT-3′; primer 2,5′-GTAATTGATCTGGGTGGTGA-3′; Primer 3,5′-GATGTTAATGATTGGGTTGG-3′; primer 4,5′-CAATTTAGATTCAGCATGAA-3′. The PCR procedure was performed with the following three combinations of the primers: primer 1 and primer 2, whose amplified product size is 1,436 bp; primer 1 and primer 3, whose amplified product size is 780 bp; and primer 1 and primer 4, whose amplified product size is 148 bp.

The PCR reaction was carried out in a total volume of 20 μl containing 1.5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.3), 200 μM each of dATP, dGTP, dTTP and dCTP (Amersham, Arlington Heights, Ill.), 1 μM of each primer set, and 1 U of Taq DNA polymerase (Perkin-Elmer, Foster City, Calif.). PCR amplifications were for 30 cycles consisting of denaturation at 92° C for 30 s, an-

nealing at 54° C for 90 s, and extension at 72° C for 150 s. The reaction products were analysed by electrophoresis on 1% agarose gel containing 0.5 μg/ml ethidium bromide. The identify of the PCR products was confirmed by their predicted size in agarose gel electrophoresis.

Fresh specimens of normal stomach (n=5), normal colon (n=5) and gall bladder (n=4) with gall stones were obtained at surgery and fresh specimens of normal pancreas (n=5) were obtained at autopsy. These were used for assay of enzyme activity or concentration. In brief, the mucosae of the stomach, gall bladder and colon were stripped. The mucosa and pancreas were homogenized with lysis buffer (Tris-HCl pH 7.4, 1 mM EDTA). The homogenate was centrifuged at 20,000 rpm for 15 min, and the supernatant was collected. Activity of pancreatic α-amylase, lipase and chymotrypsin was assayed according to the method of our previous study [33]. Concentration of trypsinogen was measured by radioimmunoassay according to the method of our previous study [33]. Fisher's exact test was used for statistical analysis, with a significant level of P<0.05.

Results

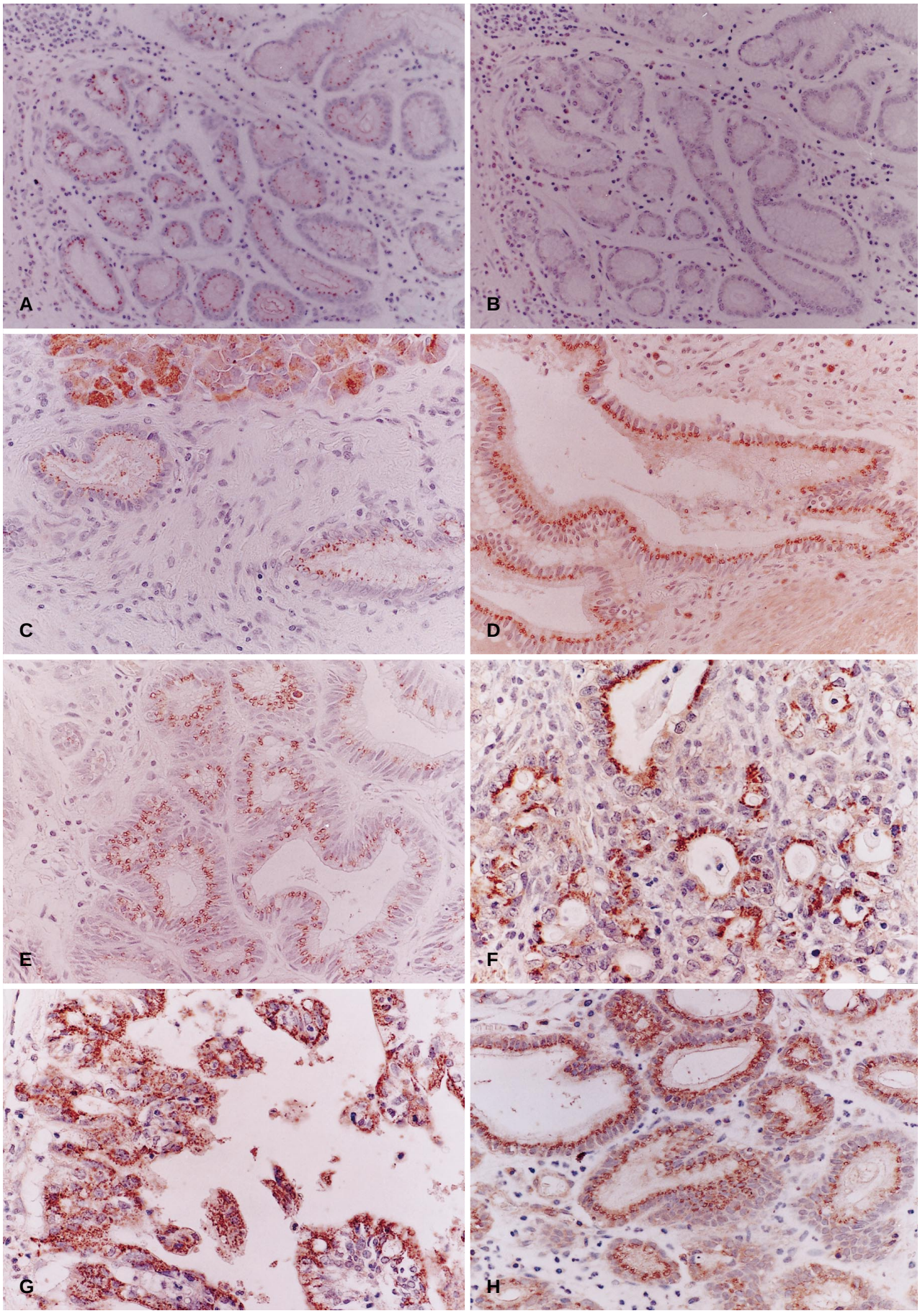
The epithelial expression of the pancreatic digestive enzymes determined immunohistochemically in normal tissues is summarized in Table 1. Immunoreactivities of pancreatic α-amylase, trypsin, chymotrypsin and pancreatic lipase were homogeneously negative (–) in all epithelial cell types of the oesophagus, small intestine and colon. The four enzyme immunoreactivities were homogeneously strongly positive (+++) in the exocrine pancreatic acinar cells (Fig. 1C).

In the salivary glands, duct cells were heterogeneously positive (++) for trypsin but homogenously negative (–) for other enzymes. The acinar cells of the salivary glands were consistently negative (–) for the four enzymes. In the stomach, expression of the pancreatic digestive enzymes was observed in 44–75% of cases. In positive cases, these enzymes were located in the glands (fundic and pyloric glands) and also in the foveolae and surface epithelium (Fig. 1A). Intestinal metaplastic epithelia in the stomach also expressed these enzymes. The immunoreactivity was heterogeneously positive (++) in positive cases. In the duodenum, immunoreactive pancreatic digestive enzymes were noted in 38–100% of cases. They were expressed in crypts and in Brunner's glands, and the immunoreactivity was heterogeneously positive (++) in positive cases. In the pancreas, large pancreatic ducts were heterogeneously positive (+++) for the four enzymes (Fig. 1C), while small pancreatic ducts were negative (–). In the gall bladder, immunoreactive pancreatic digestive enzymes were present in 33–67% of cases (Fig. 1D), and their expression was heterogeneously positive (+) in positive cases. The extrahepatic bile duct also expressed these enzymes in 40–100% of cases, and their expression was heterogeneously positive (+++) in positive cases. In any positive specimens of nonpancreatic organs, the reactivity of the four enzymes was weak and granular and located in the supranuclear cytoplasm, corresponding to Golgi apparatus (Fig. 1A, C, D), while in the pancreatic acinar cells it was strong and granular and located diffusely in the entire cytoplasm (Fig.1C). The ratio of positive cases in such organs was lower in pancreatic α-amylase than in the other three enzymes (Table 1). Histologically, these enzyme-positive epithelial cells were not heterotopic pancreatic acinar cells.

The frozen sections of normal stomach and normal pancreas showed that the epithelium was immunoreactive for pancreatic digestive enzymes heterogeneously (++) and homogeneously (+++), respectively (Fig. 3C). In contrast, the epithelial cells of normal colon never expressed the pancreatic digestive enzymes.

The expression of the enzymes in pathologic tissues is shown in Table 2. In sialoadenitis of the salivary glands, expression of trypsin was heterogeneously (++) noted in duct cells, but not in acinar cells. Trypsin expression was also heterogeneously positive (++) in tumour cells (duct cells) of pleomorphic adenomas, but negative (–) in an acinic cell tumour. One case of oesophageal squamous cell carcinoma expressed trypsin heterogeneously (+). In the stomach, adenoma (Fig. 1E) and adenocarcinoma (Fig. 1F) expressed the four pancreatic digestive enzymes relatively frequently and the immunoreactivity was heterogeneously positive (++) in positive cases. In the pancreas, ductal adenocarcinoma freququently expressed the four enzymes, and the expression was heterogenously positive (+) in positive cases. In the gall bladder, expression of the four enzymes was frequent in chronic cholecystitis, adenoma and adenocarcinoma, and was heterogeneously positive (++) in positive cases. The four pancreatic digestive enzymes were frequently expressed in adenocarcinoma of the extrahepatic bile duct (Fig. 1G), and the expression was heterogeneously positive (++). In the colon, adenoma (Fig. 1H) and adenocarcinoma occasionally expressed these enzymes, and the expression was heterogeneously positive (++) in positive cases. Immunoreactivities of the four enzymes were granular. The location of the immunoreactivity was the supranuclear cytoplasm, corresponding to Golgi apparatus in adenoma and well-differentiated carcinoma, while

Fig. 1A–H Expression of pancreatic digestive enzymes in normal and abnormal epithelium. **A** Pancreatic lipase is expressed weakly in the supranuclear cytoplasm of the normal stomach. Immunostaining for pancreatic lipase, ×280. **B** Serial section of **A**. Immunoreactivity of pancreatic lipase in the normal stomach is abolished by the absorption test. Immunostaining using anti-pancreatic lipase antibody absorbed by pancreatic lipase, ×280. **C** Pancreatic lipase is present in the normal large pancreatic ducts. Pancreatic acinar cells strongly express pancreatic lipase with granular pattern (*above*). Immunostaining for pancreatic lipase, ×230. **D** Pancreatic α-amylase is expressed in the epithelium of the normal gall bladder. Immunostaining for pancreatic α-amylase, ×230. **E** Tubular adenoma cells of the stomach express pancreatic lipase with a granular pattern in the supranuclear cytoplasm. Immunostaining for pancreatic lipase, ×230. **F** Adenocarcinoma cells of the stomach express trypsin with a granular pattern in the supranuclear cytoplasm. Immunostaining for trypsin, ×230. **G** Adenocarcinoma cells of the extrahepatic bile duct express trypsin with a granular pattern in the cytoplasm. Immunostaining for trypsinogen, ×280. **H** Tubular adenoma cells of the colon express trypsin with a granular pattern in the supranuclear cytoplasm. Immunostaining for trypsin, ×280 ▶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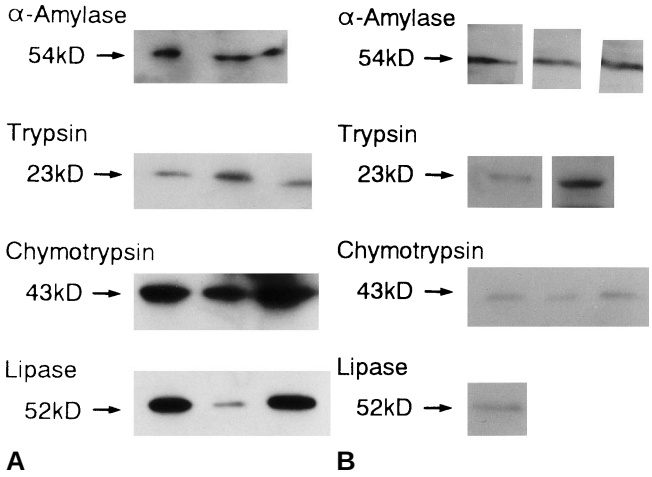

Fig. 2 Western blotting of **A** normal pancreas and **B** normal stomach. **A** When 100 μg protein is applied, a distinct band of each enzyme is recognizable. **B** When 1500 μg protein is applied, a discrete band of each enzyme is seen

it was all the cytoplasm in poorly differentiated carcinoma.

Pancreatic acinar cells were consistently and strongly positive for the four pancreatic digestive enzymes in the entire cytoplasm (Fig. 1C). The immunoreactivity in the pancreas was much stronger and more diffuse than that in the nonpancreatic tissues (Figs. 1C). No staining was seen when nonimmune mouse serum or PBS was substituted for the primary antibodies. Immunostaining using antibody solution absorbed by the pancreatic digestive enzymes consistently resulted in no staining (Fig. 1A, B). Immunostaining using the irrelevant mouse monoclonal antibodies at the same protein concentration as the antibodies against pancreatic digestive enzymes consistently resulted in no staining. Western blot analysis of normal pancreas showed specific band of each pancreatic enzyme at both 100 μg and 1500 μg protein applications; at 54 kDa for α-amylase, at 23 kDa for trypsin, at 43 kDa for chymotrypsin, and at 52 kDa for pancreatic lipase (Fig. 2A).

When 100 μg protein was applied for Western blot analysis of the normal stomach no bands were obtained. However, when 1500 μg protein was applied, a specific band of α-amylase (54 kDa), trypsin (23 kDa), chymotrypsin (43 kDa) or lipase (52 kDa) was obtained (Fig. 2B). The band of α-amylase was observed in all specimens (6/6, 100%), that of trypsin in 2/6 (33%), that of chymotrypsin in 3/6 (50%), and that of pancreatic lipase in 1/6 (17%).

In the normal stomach, specific signals for pancreatic α-amylase in mRNA using antisense probes were frequently found in the cytoplasm, the foveolar epithelium and, occasionally, the cytoplasm of the glandular epithelium (Fig. 3A, D). Signals were also found in the foveolar lumen and in the secretory area of the surface epithelial cells (Fig. 3A, D). Negative control sections using sense probes showed no signals (Fig. 3B). Immunohistochemistry of the frozen serial sections revealed the presence of immunoreactive pancreatic α-amylase in the cytoplasm of foveolar and glandular epithelium (Fig. 3C). However, no signals were found in the epithelium of the normal colon. In the normal pancreas, a large number of specific signals using antisense probes were found in the cytoplasm of the acinar cells, and negative control sections using sense probes showed no signals. The signals in the pancreas were much more numerous than those in the stomach.

Figure 4 shows the results of RT-PCR for pancreatic α-amylase mRNA in two cases of normal gastric tissue. In both cases, three complementary DNA fragments of approximate sizes of 1.5 kbp, 0.8 kbp and 150 bp were amplified. The sizes of these fragments were compatible with their predicted sizes. These results suggest that mRNA of pancreatic α-amylase is expressed in normal gastric tissue.

In normal stomach, normal pancreas and gall bladder with cholecystitis, enzyme activity or concentration was present in all cases (Table 3). Activity and concentration in the pancreas were much higher than in the stomach and gall bladder ($P<0.01$). In contrast, there was little or no enzyme activity or protein in the normal colon comparted with that in the normal stomach and gall bladder with cholecystitis ($P<0.05$). There was no statistical difference in their activity between normal stomach and gall bladder with cholecystitis ($P>0.05$).

Table 3 Activity and concentration of pancreatic digestive enzymes in the normal pancreas, normal stomach, normal colon and gall bladder with cholecystitis

Case no.	Pancreatic α-amylase	Trypsin	Chymotrypsin	Lipase
Normal pancreas				
1.	1.5 IU/g	545 ng/g	8.2 U/g	26.5 U/g
2.	1.2 IU/g	323 ng/g	7.7 U/g	16.5 U/g
3.	2.1 IU/g	639 ng/g	9.2 U/g	30.3 U/g
4.	1.3 IU/g	256 ng/g	11.3 U/g	17.8 U/g
5.	3.2 IU/g	221 ng/g	5.8 U/g	20.4 U/g
Normal stomach				
1.	0.05 IU/g	27.2 ng/g	0.05 U/g	0.12 U/g
2.	0.02 IU/g	15.3 ng/g	0 U/g	0.06 U/g
3.	0.08 IU/g	18.4 ng/g	0.04 U/g	0.09 U/g
4.	0.06 IU/g	32.6 ng/g	0.09 U/g	0.11 U/g
5.	0.02 IU/g	28.9 ng/g	0.06 U/g	0.04 U/g
Normal colon				
1.	0.01 IU/g	2.4 ng/g	0 U/g	<0.01 U/g
2.	<0.01 IU/g	2.8 ng/g	0 U/g	0.01 U/g
3.	0.01 IU/g	1.1 ng/g	0 U/g	<0.01 U/g
4.	<0.01 IU/g	5.4 ng/g	<0.01 U/g	0.02 U/g
5.	0.02 IU/g	3.3 ng/g	0.01 U/g	0.03 U/g
Gall bladder with cholecystitis				
1.	0.01 IU/g	18.9 ng/g	<0.01 U/g	0.02 U/g
2.	0.03 IU/g	10.0 ng/g	0.08 U/g	0.05 U/g
3.	0.05 IU/g	21.1 ng/g	0.02 U/g	0.16 U/g
4.	0.06 IU/g	9.6 ng/g	0 U/g	0.12 U/g

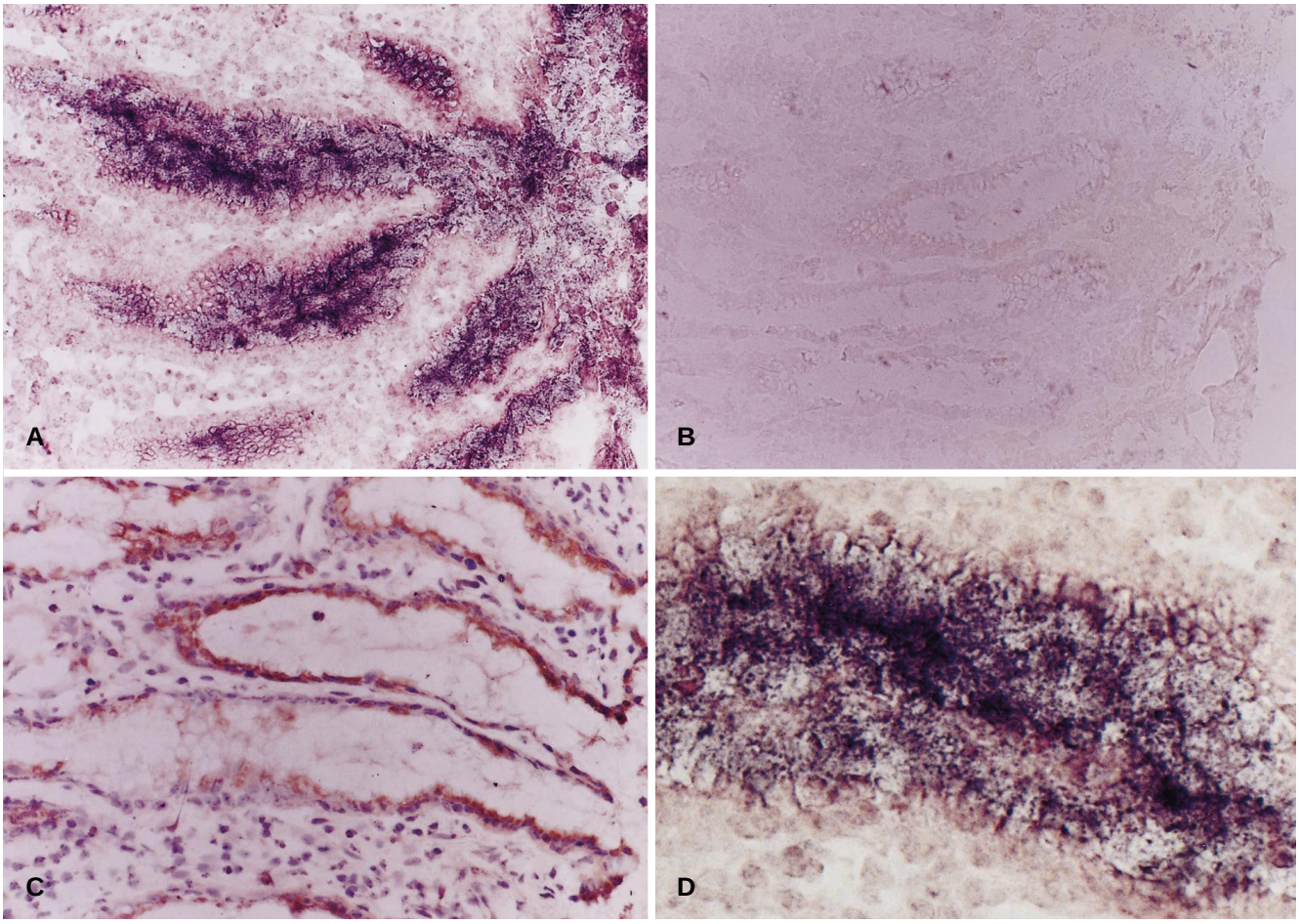

Fig. 3A–D In situ hybridization for mRNA of pancreatic α-amylase using antisense probes shows many signals (*blue*) in the cytoplasm of the foveolar and surface epithelia cells and also in the secretory areas (foveolar lumina and gastric luminal surface) of the stomach (**A**), while that using sense probes shows no signals (**B**). Immunohistochemistry of a frozen serial section shows immunoreactive pancreatic α-amylase (*brown*) in the cytoplasm of the foveolar epithelial cells (**C**). **A, B, C** are semiserial sections of the same area, ×320. **D** Higher magnification shows that mRNA of pancreatic α-amylase (*blue*) is located in the cytoplasm and in the lumen of a foveola of the normal stomach. ×850

Discussion

There have been few descriptions of pancreatic digestive enzymes in nonpancreatic tissues. Our recent studies have shown immunoreactive pancreatic digestive enzymes in the intrahepatic biliary system [26–30, 32]. Although lipase activity has been noted in several tissues, including the stomach (gastric lipase) [1, 3, 16, 17, 25, 34], pancreatic lipase has not been reported in non-pancreatic organs. Donaldson et al. [5] reported amylase activity in the human bile, and recently Doglioni et al. [4] reported that pancreatic acinar metaplasia in the stomach was positive for immunoreactive pancreatic α-amylase and lipase. There have been no systematic reports on the in situ expression of pancreatic digestive enzymes in nonpancreatic tissues.

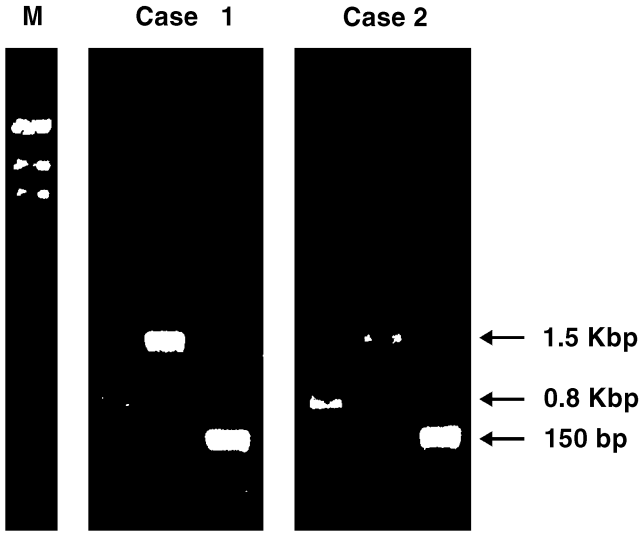

Fig. 4 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis using three sets of primers specific for pancreatic α-amylase DNA. Amplified complementary DNA fragments were detected by electrophoresis in 1% agarose gel. The complementary DNA fragments amplified by RT-PCR using the combination of primers 1 and 2 (predicted size is 1436 bp), primers 1 and 3 (predicted size is 780 bp), and primers 1 and 4 (predicted size is 148 bp) were detected in middle lane, left lane and right lane of each case, respectively. The RT-PCR analysis shows three specific bands (approximately 1.5 kbp, 0.8 kpb and 150 bp) in two cases of normal stomach (*M* molecular marker)

In immunohistochemistry, the specificity of the immunorectivity examined must be defined. We applied various methods to confirm the specificity, and it is very likely that each immunostaining is specific for a particular pancreatic digestive enzyme. Western blot analysis showed specific signals of the pancreatic digestive enzymes in normal pancreas, thus confirming the specificity of the antibodies. The antibody against pancreatic lipase recognizes a 52-kDa protein [33], suggesting that the antibody reacts with pancreatic lipase but not with other lipases of different molecular weight. The antibody to pancreatic α-amylase does not react with salivary α-amylase [32]. In situ hybridization and RT-PCR analysis for pancreatic α-amylase mRNA and the assay of pancreatic enzyme activities and concentration in the present study also strongly suggest that the immunoreactivity is specific for each pancreatic digestive enzyme.

We found that normal epithelial cells of the salivary glands, stomach, duodenum, gall bladder, extrahepatic bile duct, and large pancreatic ducts expressed immunoreactive pancreatic α-amylase, trypsin, chymotrypsin and pancreatic lipase. The immunoreactivities of these enzymes were granular and located in the supranuclear cytoplasm corresponding to the Golgi apparatus. The enzyme-positive cells were not ectopic pancreatic acinar cells histologically. These findings show that epithelial cells of the salivary gland, stomach, duodenum, gall bladder and large pancreatic ducts contain these enzymes, and suggest that they produce them and secrete them into the gut lumina. However, it is clear that, nonpancreatic organs produce and secrete much smaller quantitites of pancreatic enzymes than does the pancreas. Some cases were negative for the enzymes in the stomach, duodenum and gall bladder, and it is likely that the amount of pancreatic digestive enzymes was too small to be detected by immunohistochemistry.

Western blotting showed a specific signal for each pancreatic digestive enzyme in the normal stomach, though the incidence varied with the enzymes. In situ hybridization showed the presence of mRNA of pancreatic α-amylase in the normal stomch, which was positive for immunoreactive pancreatic α-amylase. The mRNA of pancreatic α-amylase was absent from the normal colon, which was consistently negative for immunoreactive pancreatic α-amylase. The RT-PCR analysis strongly suggests the presence of pancreatic α-amylase mRNA in the normal stomach positive for immunoreactive pancreatic α-amylase. It is therefore very likely that the gastrointestinal epithelial cells positive for immunoreactive pancreatic digestive enzymes do produce pancreatic digestive enzymes. Assay of enzyme activity or concentration revealed that the normal stomach, gall bladder and pancreas possessed this enzyme activity or proteins, while the normal colon did not (or showed only a little). Our findings strongly suggest the presence of pancreatic digestive enzymes in epithelial cells positive by immunoreactivity and their absence in nondecorated cells.

There have been a few observations of the activity of pancreatic enzymes in the epithelial cells of nonpancreatic organs [1, 3–5] and some ultrastructural observations of zymogen granules in these cells [6, 18]. However, we find the presence of pancreatic digestive enzymes in several nonpancreatic gastrointestinal organs, albeit at much lower levels than in the pancreas, confirming that the nonpancreatic organs produce much smaller quantities of pancreatic digestive enzymes than does the pancreas. The secretory mechanism of the pancreatic digestive enzymes may be different between nonpancreatic organs and the pancreas: for example, pancreatic digestive enzymes do not accumulate in the cytoplasm as zymogen granules, but may be directly secreted ino gut lumina via membrane-bounded exocytosis in nonpancreatic tissues. Detailed studies using immunoelectron microscopy may be necessary to evaluate the secretory process. The activation mechanism of the pancreatic proenzymes in the nonpancreatic organs also remains to be elucidated.

The expression of these enzymes may have important implications for the cell lineage. In the present study, immunoreactive pancreatic digestive enzymes were present exclusively in organs arising from embryonic foregut, and the common cell lineage of the pancreas and the liver has been examined in experimental animals [13, 15, 22] and in humans [31, 36]. The distribution pattern of pancreatic digestive enzymes in the present study further confirms that the stomach, duodenum, gall bladder, extrahepatic bile duct and large pancreatic ducts have a common cell lineage with the exocrine pancreas.

Benign and malignant neoplasms arising from epithelial cells normally expressing the pancreatic digestive enzymes frequently contained immunoreactive pancreatic α-amylase, trypsin, chymotrypsin and pancreatic lipase. In contrast, neoplastic epithelia deriving from pancreatic digestive enzyme-negative normal epithelial cells, such as colon and oesophagus, only occasionally expressed these enzymes. This suggests that some epithelia neoexpress pancreatic digestive enzymes after neoplastic transformation. In general, there are malignant tumours that abberrantly express digestive enzymes with serum elevation [2, 21, 37]. The majority of such cases produce salivary α-amylase [2, 21]. Only two cases with tumour cells expressing pancreatic α-amylase with serum hyperamylasemia have been reported [11, 35]. The present study revealed that benign and malignant neoplasms of the gastrointestinal tract may contain immunoreactive pancreatic digestive enzymes. It seems that more neoplasms produce pancreatic digestive enzymes than was previously recognized. However, it is not clear that the pancreatic digestive enzymes will enter the bloodstream and cause elevation of the serum levels.

Pancreatic digestive enzymes have been shown to be present in acinar cell carcinoma of the pancreas and pancreatoblastoma, but not in ductal adenocarcinoma [7, 8, 14, 19, 24]. However, Kim et al. [12] demonstrated pancreatic digestive enzymes (lipase and trypsin) in about 17% of pancreatic ductal carcinomas. In addition, Ohta et al. [20] demonstrated immunoreactive pancreatic enzymes (pancreatic α-amylase, trypsinogen and lipase) in normal human pancreatic ducts. The present study also

showed immunoreactive pancreatic digestive enzymes in the normal pancreatic ducts and in pancreatic ductal carcinomas, supporting the findings of Kim et al. [12] and Ohta et al. [20].

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