Discrimination between chronic pancreatitis and pancreatic carcinoma can be complicated, particularly in brush cytology specimens. Previous studies have shown that the oxygen insensitivity of the histochemical reaction to detect glucose-6-phosphate dehydrogenase activity based on neotetrizolium reduction can be used for discriminating malignant cells from nonmalignant cells. In the present study, we investigated the value of the assay for differential diagnosis between the two pancreatic diseases.

INTRODUCTION

The clinical presentation of chronic pancreatitis often resembles that of pancreatic cancer (1, 2). Differential diagnosis cannot always be obtained with certainty (3–5). Moreover, it can be difficult to discriminate cells in biopsies or brush cytology specimens of chronic pancreatitis from those of pancreatic cancer on the basis of the number of nucleoli, nuclear contour irregularity, nuclear hyperchromasia, coarse chromatin, and nucleus:cytoplasm ratio (6). Therefore, more reliable preoperative discriminators between chronic pancreatitis and pancreatic malignancy are needed.

Activity of G6PDH increases in early stages of carcinogenesis and often precedes morphological changes (7–9). G6PDH is a housekeeping enzyme that regulates the pentose phosphate pathway, and its main role in metabolism is to provide AADPH for biosynthesis and detoxification (10). G6PDH activity can be demonstrated histochemically in cryostat sections or cytology specimens by using a tetratrizolium salt as final electron acceptor to produce intensely colored formazan (10). Because G6PDH activity can be high in any proliferating cell (11), its activity can be demonstrated histochemically in cryostat sections or cytology specimens by using a tetrazolium salt as final electron acceptor to produce intensely colored formazan. Activity of G6PDH increases in early stages of carcinogenesis and often precedes morphological changes (7–9). G6PDH is a housekeeping enzyme that regulates the pentose phosphate pathway, and its main role in metabolism is to provide AADPH for biosynthesis and detoxification (10). G6PDH activity can be demonstrated histochemically in cryostat sections or cytology specimens by using a tetratrizolium salt as final electron acceptor to produce intensely colored formazan (10). Because G6PDH activity can be high in any proliferating cell (11), its activity

oxygen insensitivity test is a simple and valuable tool in addition to conventional pathology for differential diagnosis between pancreatic cancer and pancreatic cancer, both in biopsies and in brush cytology specimens.

ABSTRACT

Discrimination between chronic pancreatitis and pancreatic carcinoma can be complicated, particularly in brush cytology specimens. Previous studies have shown that the oxygen insensitivity of the histochemical reaction to detect glucose-6-phosphate dehydrogenase activity based on neotetrizolium reduction can be used for discriminating malignant cells from nonmalignant cells. In the present study, we investigated the value of the assay for differential diagnosis between the two pancreatic diseases. Oxygen insensitivity in ductal epithelial cells in normal human pancreas, chronic pancreatitis, and pancreatic carcinoma was determined by quantitative image analysis in sections of biopsies and in brush cytology preparations. In sections, the reaction in the absence of oxygen was a proper reflection of glucose-6-phosphate dehydrogenase activity, whereas in the presence of oxygen only malignant cells showed a significant reaction. Of 39 brush cytology specimens, diagnosis of all 11 cases of pancreatitis and 28 cases of cancer with the oxygen insensitivity test were in agreement with independent measures of chronic pancreatitis and cancer. The oxygen insensitivity test is a simple and valuable tool in addition to conventional pathology for differential diagnosis between pancreatic cancer and pancreatic cancer, both in biopsies and in brush cytology specimens.

Materials and Methods

Tissue Specimens. Biopsies of chronic pancreatitis, pancreatic carcinoma, and normal pancreas were obtained from the Department of Surgery in the Academic Medical Center (Amsterdam, the Netherlands). Seven specimens of normal pancreas (Fig. 1A) were obtained from patients who underwent partial pancreatectomy during resection of other types of lesions. Surgical specimens from 23 patients with chronic pancreatitis and from 17 patients with pancreatic cancer who underwent pancreatic resections between 1995 and 1997 were reviewed microscopically by a pathologist (G. J. A. O.) in a blinded fashion for the presence of (atypical) hyperplasia or malignancy (Fig. 1B). Ductal epithelial hyperplasia was defined as an abnormal increase in the number of cells lining pancreatic ducts, which are more than twice as tall as normal cells (19). Hyperplasia varied from simple to atypical. Simple ductal hyperplasia was characterized by basally located uniform nuclei, cellular crowding, with focal pseudostratification of the nuclei. Papillary mucinous hyperplasia was recognized by papillary structures with tufting of epithelial fronts into the ductal lumen and a core of fine fibrovascular tissue derived from the lamina propria. Atypical hyperplasia was established when all columnar epithelium formed a papillary structure without a fibrous core and was considered as a precancerous condition or carcinoma in situ (19). Simple ductal hyperplasia was observed in 12 specimens of chronic pancreatitis and 4 specimens of pancreatic carcinoma. Papillary hyperplastic ducts were observed in three specimens of chronic pancreatitis and three specimens of pancreatic carcinoma. In these three specimens of chronic pancreatitis, atypical hyperplasia was observed as well. Features that characterize chronic pancreatitis (Fig. 1C), i.e., atrophy of exocrine acinar tissue and abundant fibrosis and connective tissue, were observed in the vicinity of almost every carcinoma (Fig. 1B).

Brush cytology preparations were obtained postoperatively from 39 patients with proven or suspected carcinoma of the pancreatic head region who underwent a Whipple resection (subtotal pancreatico-duodenectomy) between 1996 and 1998. The distal common bile duct and the main pancreatic duct were brushed with an endocervical brush (Cervibrush; Cellpath, Hemel Hempstead, United Kingdom) immediately after arrival of the resection specimens at the histology laboratory.

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The cytology specimens were collected in a blinded fashion, and results of the oxygen insensitivity test were compared afterward with the diagnosis of the pathologist of the cytology specimens on the one hand and histology on the other. Because carcinomas of the ampulla of Vater have a better prognosis than the other types of cancer in the pancreatic head region (pancreas or distal bile duct; Ref. 20), the residual activity of brush cytology specimens was analyzed both as one group and when subdivided in groups dependent on tumor origin.

**Tissue and Cell Processing.** Fresh tissue biopsies were immediately frozen in liquid nitrogen. The frozen material was stored at −80°C until further use. Serial sections (8 μm thick) were cut at −25°C on a motor-driven cryostat (Bright, Huntingdon, United Kingdom) fitted with a retraction microtome at a low but constant speed to minimize variation in section thickness (21). Sections were stored at −80°C until use. Brush cytology preparations of epithelial cells on glass slides were made by smearing pancreatic duct brushings of fresh pancreatic tissue and were stored at −80°C until use.

**Oxygen Insensitivity Test Based on the Histochemical Assay of G6PDH Activity.** Incubation media to demonstrate G6PDH activity were prepared as described previously in detail (16, 21). Incubation media were poured into glass vials and equilibrated for 10 min in an atmosphere of either 100% oxygen or 100% nitrogen using a tonometer to avoid formation of gas bubbles in the viscous media. Meanwhile, sections and cytological smears were air dried (5 min at 37°C). Plastic rings were placed around sections or cells and filled with media. Coverslips were placed upon the rings to avoid loss of gas from the media during incubation. Serial sections and cell smears were incubated in triplicate in the presence of oxygen or nitrogen. Serial sections were obtained from opposite sides of all biopsies. In this way, we were able to establish that variation in oxygen insensitivity within biopsies was negligible. In case of brush cytology specimens, part of each cell smear was incubated in the presence of oxygen, and the remainder was incubated in the absence of oxygen. After 10 min of incubation, sections or smears were rinsed thoroughly and mounted in glycerol jelly. Reproducibility of the histochemical reaction was tested by inclusion of at least two samples in a run that were shown to be oxygen insensitive in a previous run. Control reactions were performed by omitting substrate and coenzyme from the incubation media (21). No or negligible amounts of formazan were produced in these controls, which implied that the quantitative histochemical method for the detection of G6PDH activity was specific.

**Image Analysis and Processing.** End point absorbance measurements of formazan production by G6PDH activity in epithelial cells were performed with image analysis as described previously in detail (22, 23) using a Vanox-T photomicroscope (Olympus, Tokyo, Japan) with a ×2 objective (N.A. 0.08), monochromatic light of 585 nm, a Cohu 4913 CCD camera (Cohu, San Diego, CA), an 8-bit monochrome frame grabber (LG-3; Scion, Frederick, MD), and a Power Macintosh 8100/110 computer (Apple, Cupertino, CA) using the public domain NIH Imaging software program (written by Wayne Rasband; NIH Image, U.S. National Institutes of Health, Bethesda, MD).
RESULTS

Biopsies. The histochemical assay in the absence of oxygen showed that ductal epithelial cells contained highest G6PDH activity of all cell types present in the biopsies (Fig. 1, D–F). G6PDH activity in ductular epithelial cells was not significantly different in normal pancreas (mean ± SD, 3.2 ± 1.1), chronic pancreatitis (mean ± SD, 4.8 ± 3.0), and pancreatic carcinomas (mean ± SD, 4.5 ± 1.6). In the presence of 100% oxygen, residual activity was always <20% in epithelial cells of ducts in normal pancreas (mean percentage ± SD, 4.4 ± 3.2; Figs. 1G and 2). Residual activity in epithelial cells of ducts in chronic pancreatitis was also <20% in 20 of 23 biopsies (mean percentage ± SD, 8.1 ± 5.2; Figs. 1H and 2). Residual activity in pancreatic carcinoma (mean percentage ± SD, 57.2 ± 23.6; Figs. 1H and 2) was significantly higher than that in normal pancreas and chronic pancreatitis (P < 0.001). In fact, all carcinomas showed residual activity ≥35% (Fig. 2).

The 20 biopsies of chronic pancreatitis with residual activity <20% contained only histologically normal and simple hyperplastic ducts but no papillary hyperplastic ducts. Each biopsy of the three remaining chronic pancreatitis patients (patients A, B, and C) showed residual activity >20% in a single atypical hyperplastic duct (Fig. 2). In the biopsy of patient A, an atypical hyperplastic duct was the only oxygen-insensitive duct, with a residual activity of 38%. In the biopsy of patient B, one oxygen-insensitive atypical hyperplastic duct and one oxygen-insensitive papillary hyperplastic duct were found with residual activities of 35 and 72%, respectively (Fig. 3). The biopsy of patient C contained one atypical hyperplastic duct with residual activity of 30% and a single mucinous cystadenoma with a residual activity of 46%. The remainder of the biopsies of patients A, B, and C was oxygen-sensitive, similar to other biopsies of chronic pancreatitis.

Patients A and B appeared to have pancreatic malignancy afterward. Malignancy was not found by the pathologist in the biopsies that were used for the oxygen insensitivity test. Patient A died shortly after diagnostic confirmation of pancreatic cancer. All other 22 patients with chronic pancreatitis were still alive up to 2 years of follow-up. Patient B is considered to be an exception, because various neoplasms, such as colorectal carcinoma and adenomatous polyps, were demonstrated at different time points during the last 20 years. In summary, oxygen insensitivity was only observed in malignant ductal epithelium or in hyperplastic epithelium in patients with proven neoplasms.

Brush Cytology Preparations. The oxygen insensitivity test was applied in a blinded fashion to brush cytology preparations of 39 patients with proven or suspected carcinoma in the pancreatic head region (Fig. 4). All patients that were diagnosed on the basis of brush cytology specimens and histology to have a malignancy after pancreatic-duodenectomy (n = 27) showed oxygen-insensitive neoplastic epithelial cells [mean residual activity (%) ± SD, 73.1 ± 16.6]. No differences in residual activity were observed between carcinomas in the pancreas (mean percentage ± SD, 70.6 ± 17.3), distal bile ducts (mean percentage ± SD, 74.0 ± 16.8), and ampulla of Vater (mean percentage ± SD, 76.6 ± 17.7).

Eleven patients with suspected carcinomas showed oxygen-sensitive epithelial cells (mean percentage ± SD, 14.3 ± 4.3). These patients were diagnosed to have chronic pancreatitis both after examination of the brush cytology specimens and histology by the pathologist. The residual activity of these patients was significantly lower (P < 0.0001) than that of patients who were diagnosed to have malignancy. One patient, who was diagnosed by the pathologist on the basis of examination of brush cytology specimens to have chronic pancreatitis, showed a residual activity of 72.0%. This patient was diagnosed later on the basis of histology to have a carcinoma in the pancreatic head.

DISCUSSION

The histochemical assay with the use of NT gives a proper reflection of actual G6PDH activity provided that the assay is performed in the absence of oxygen (10, 15). Under these conditions, epithelial cells of normal, hyperplastic, and cancerous ducts showed similar G6PDH activity. In the presence of oxygen, NT-formazan production was inhibited in epithelial cells of histologically normal and simple hyperplastic ducts, because the residual activity was always <20%. Pancreatic carcinomas were always oxygen insensitive, because the residual activity was >35%. Similar results were found for colorectal
The oxygen insensitivity test thus proved to be a valid discriminator between chronic pancreatitis and pancreatic cancer. Oxygen insensitivity was observed in three biopsies of chronic pancreatitis in single atypical ducts only. Two of these patients had pancreatic malignancy, and one patient had a mucinous cystadenoma. Mucinous cystadenomas have a high malignant potential, and transition into cystadenocarcinomas is often observed (24, 25). The finding of oxygen-insensitive hyperplastic ducts in these patients may be explained in two ways: (a) the oxygen insensitivity of epithelial cells in these atypical hyperplastic ducts is attributable to the presence of malignancy in the vicinity. Elevated oxygen insensitivity in hyperplasia adjacent to carcinomas was found in the colon as well (9); and (b) because atypical hyperplasia itself is considered to be precancerous (19, 26, 27), oxygen insensitivity in these ducts may also be caused by their own progression toward malignancy. Invasive carcinoma in atypical hyperplastic ducts has occasionally been described (28). This atypical hyperplasia of the intraductal mucinous papillary type is a relatively novel and more and more often reported entity (29).

Because oxygen insensitivity was observed in all papillary and atypical hyperplastic ducts and all carcinomas, we conclude that oxygen insensitivity starts to occur in papillary hyperplasia. Simple hyperplastic ducts, which were frequently observed in chronic pancreatitis and nearby carcinomas, were always oxygen sensitive. This development of oxygen insensitivity is similar to that in human and murine colorectal carcinogenesis, where oxygen insensitivity develops during the transition from premalignancy to malignancy (9). There is little or no evidence of progression of simple hyperplastic ducts in pancreas (19) and colorectal hyperplastic polyps (28) into a carcinoma, and both are always oxygen sensitive.

The sensitivity of the test to detect cancer cells in brush cytology preparations (Table 1) was better than that of conventional cytological examination reported in the literature, which ranges from 30 to 92% (30–33). This wide range in sensitivity may be the result of variation in interpretation or numbers of cells collected (34). Furthermore, confounding may occur of atypical cells from chronic pancreatitis and benign-appearing cells from well-differentiated pancreatic carcinomas.

Table 1  Sensitivity and specificity of diagnosis by routine cytology and the oxygen insensitivity of brush cytology specimens of 39 patients with proven or suspected carcinoma in the pancreatic head region

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* Oxygen insensitivity is expressed as residual activity. Residual activity <20% is taken as nonmalignant and >20% as malignant.
Finally, pancreatic carcinomas may develop from chronic pancreatitis (36–38), which makes differential diagnosis even more complicated. However, because the brush cytology preparations in our study were obtained postoperatively, the sampling may have been directed toward suspicious sites. It remains to be determined whether the assay has the same sensitivity in the setting of more randomly assigned samplings (e.g., endoscopic retrograde pancreatic juice sampling) prior to a decision of surgical intervention.

All brush cytology specimens of carcinomas were oxygen insensitive, and all chronic pancreatitis specimens showed oxygen sensitivity of the cells without exception. The sensitivity of the oxygen insensitivity test was demonstrated by the one patient who was diagnosed by the pathologist on the basis of brush cytology specimens to have chronic pancreatitis but showed high activity of G6PDH in the presence of oxygen (Table 1). This patient appeared to have a carcinoma, as was determined by histology afterward. Therefore, the oxygen insensitivity phenomenon as a diagnostic aid in carcinoma of the pancreas. In: J. R. Patterson, L. Bitsensky, and J. Chayen (eds.), Quantitative Cytochemistry and Its Applications, pp. 241–251, New York: Academic Press, 1979.


