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Immunohistochemical demonstration of the carbonic anhydrase isoenzymes I and II in pancreatic tumours

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Summary

The location of carbonic anhydrase (CA) isoenzymes I, II and VI in normal and neoplastic pancreatic tissue was studied using polyclonal antisera and the immunoperoxidase technique. Samples were obtained from patients with well-differentiated ($n = 4$), moderately differentiated ($n = 1$) and poorly differentiated ($n = 4$) ductal adenocarcinomas, cystadenocarcinoma ($n = 2$), adenosquamous carcinoma ($n = 1$), acinar adenocarcinoma ($n = 1$), gastrinoma ($n = 3$), insulinoma ($n = 3$) and glucagonoma ($n = 1$). The control specimens were from a patient with traumatic laceration of the pancreas. The normal and malignant endocrine tissue showed intense positive staining for CA I localized in the cells expressing glucagon. In the exocrine pancreatic tissue, CA II was detected in the normal and neoplastic ductal epithelium. No specific staining was detected with anti-CA VI serum in either normal or malignant tissue.

Introduction

Carbonic anhydrase (CA; E.C. 4.2.1.1.) catalyses the reversible hydration of carbon dioxide. Three distinct cytoplasmic isoenzymes have been identified; these are known as CA I, CA II and CA III. CA I and II represent the major non-haemoglobin proteins of the erythrocyte and CA III is abundantly present in skeletal muscle (Carter, 1972; Tashian *et al.*, 1983; Tashian, 1989). CA I has relatively low enzymatic activity, while CA II is referred to as a 'high-activity' isoenzyme because of its high specific activity. Apart from the soluble isoenzymes, at least three other members of the isoenzyme family have been identified in mammals: membrane-bound CA IV, mitochondrial CA V, and salivary CA VI (Fernley *et al.*, 1979; Wistrand, 1979; Dodgson *et al.*, 1980).

CAs are probably among the most important factors regulating the acid–base balance in the gastrointestinal tract, and thereby participate in the protection of the oesophageal and gastrointestinal mucosa from acidity (Swenson, 1991). CA II is the most widely distributed CA isoenzyme and has been localized in the epithelial cells of the stomach, small intestine and colon (Kumpulainen, 1979; Lönnerholm *et al.*, 1985; Parkkila *et al.*, 1994). Furthermore, it has been shown to be present in the epithelial cells of the pancreatic and bile ducts (Kumpulainen & Jalovaara, 1981; Spicer *et al.*, 1982; Parkkila *et al.*, 1994). In the pancreatic duct cells, CA II supplies the pancreatic juice with bicarbonate, thus participating in the neutralization of the acid gastric juice delivered to the duodenum. In the gastrointestinal tract, CA I has been reported to be present only in the surface epithelial cells of the colonic mucosa (Lönnerholm *et al.*, 1985; Parkkila *et al.*, 1994) and A cells of pancreatic Langerhans islets (Parkkila *et al.*, 1994) in which its physiological function has remained unclear. The present work was undertaken to determine whether the expression of CA I and II is sustained in pancreatic endocrine and exocrine tumours.

Materials and methods

Antisera

Polyclonal rabbit antisera to human CA I and II produced and characterized by Parkkila *et al.* (1993a) and to human CA VI by Parkkila *et al.* (1991; 1993b) were used. The antisera showed no cross-reactivity in immunoblottings. Antihuman gastrin and antihuman insulin antibodies were obtained from Dakopatts (Copenhagen, Denmark) and antiporcine glucagon antibodies from Novo Research Institute (Copenhagen, Denmark).

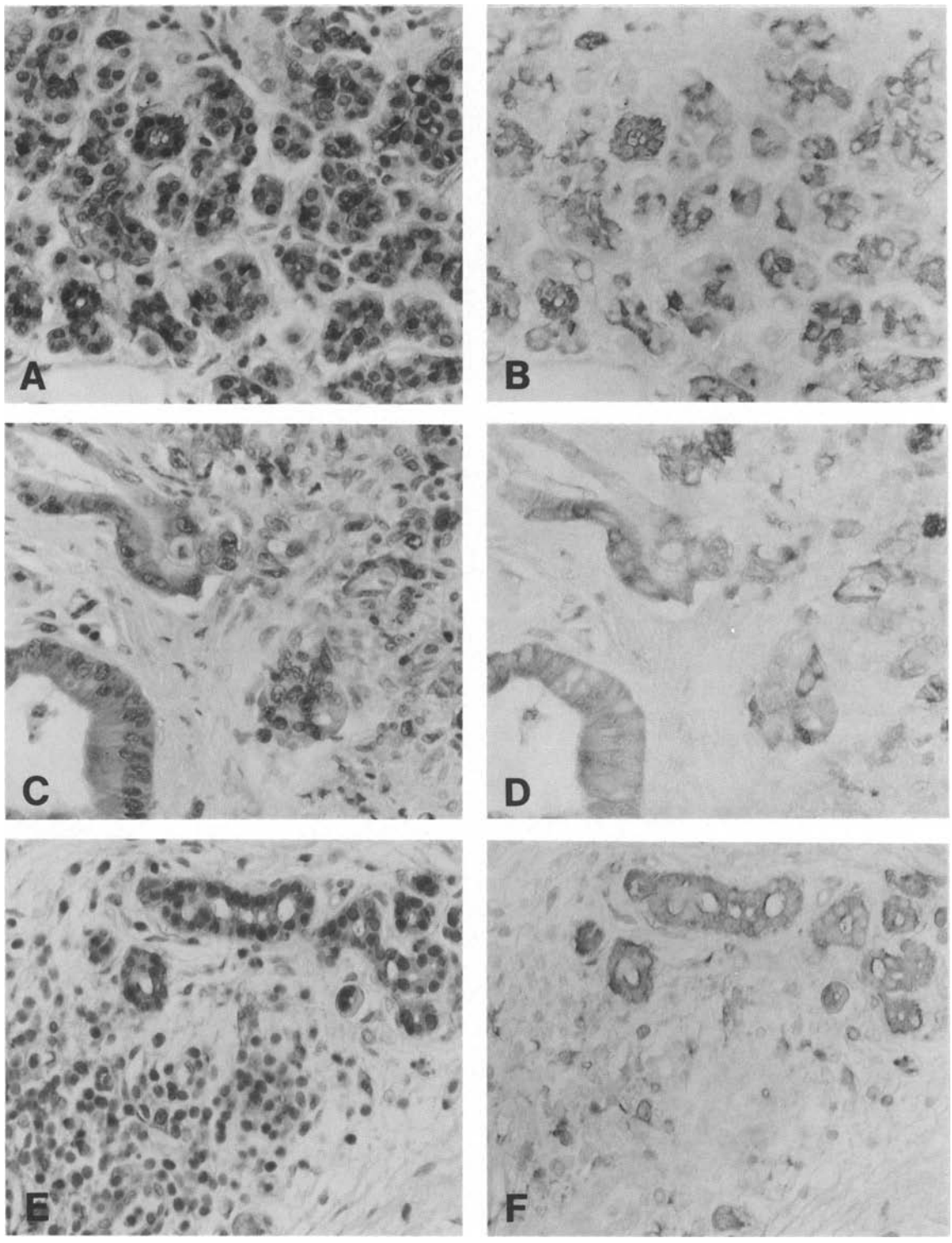

Fig. 1. A–F. *Caption on facing page*

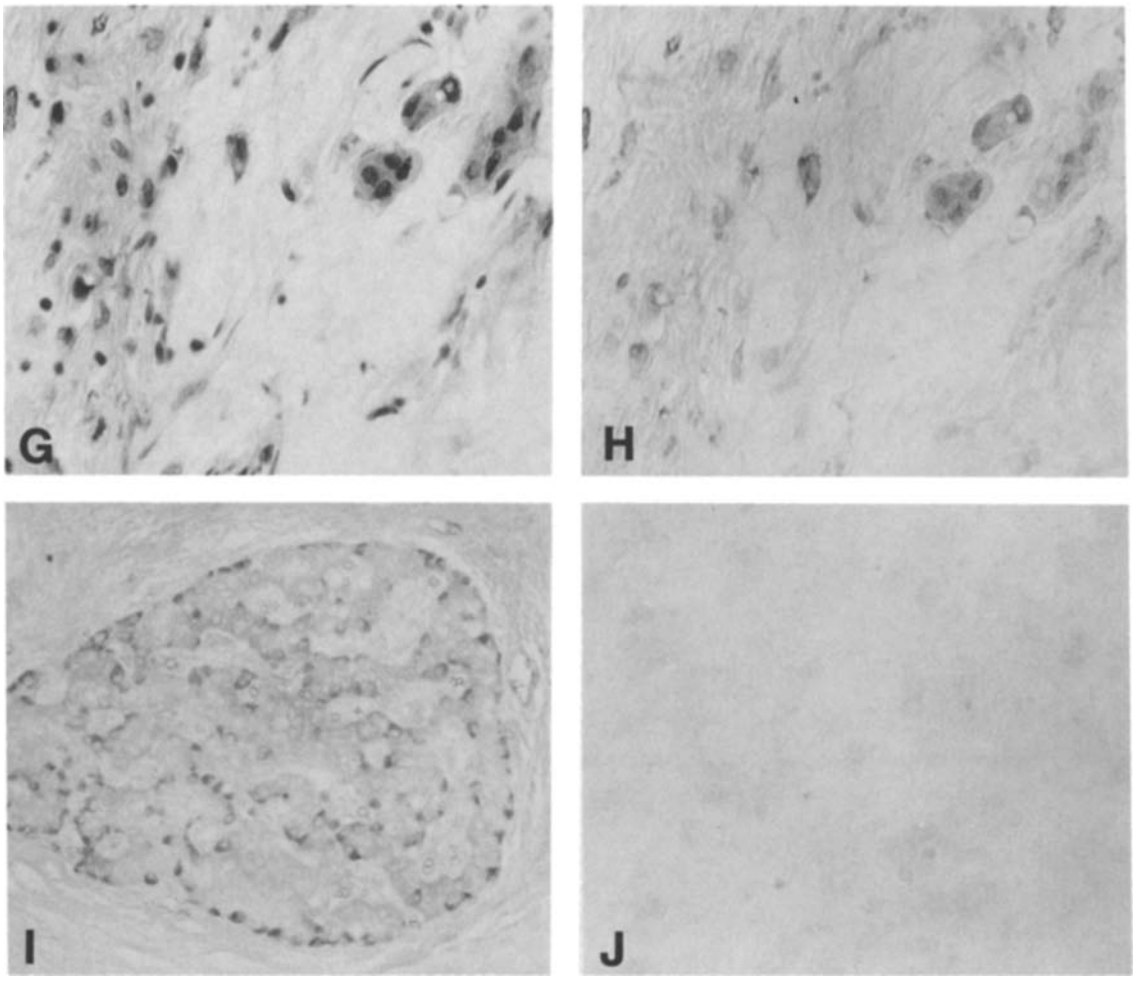

Fig. 1. A well-differentiated (A–D, I) and a moderately differentiated (E–H, J) pancreatic adenocarcinoma stained with Haematoxylin and Eosin (A, C, E, G), anti-CA II (B, D, F, H), anti-CA I (I) and anti-CA VI (J) antibodies. The Haematoxylin and Eosin counterstained sections show atypical nuclei in the malignant cells and a moderate or strong desmoplasia. Malignant epithelial cells stained with anti-CA II serum tend to form glandular structures in both cases. Langerhans islets are well-preserved and the peripheral cells show CA I-positive staining (I). The immunostaining with anti-CA VI serum is negative (J). Carnoy's fluid fixation. ×360.

Preparation of the tissues

Samples of the human pancreas were obtained alongside routine histopathological specimens taken during surgery at Oulu University Hospital. The patients were operated for pancreatic tumours ($n = 20$) and traumatic laceration of the pancreas ($n = 1$). Each tissue sample was divided into several small pieces 5–10 mm thick. The specimens were fixed for either 6 h in Carnoy's fluid (6:3:1 by vol. absolute ethanol–chloroform–glacial acetic acid) or 18 h in 4% neutral-buffered formaldehyde at 4°C as described earlier (Parkkila *et al.*, 1994). Both Carnoy's fluid and formaldehyde fixations preserved tissue morphology and enzyme antigenicity well and no difference in the staining intensity or its distribution was recognized (data not reported). The tissue specimens were dehydrated, embedded in paraffin wax in a vacuum oven at 58°C, and sections, 5 μm thick, placed on gelatine-coated microscope slides. Routine histological examination of the samples was performed after Haematoxylin–Eosin staining.

Immunohistochemistry

The sections fixed with either Carnoy's fluid or formaldehyde were stained using the immunoperoxidase technique. The steps in the staining were as follows:

1. Pre-treatment of the sections with full swine serum for 40 min and rinsing in phosphate-buffered saline (PBS).
2. Incubation either for 1 h (Carnoy's fluid-fixed specimens) or 18 h (formaldehyde-fixed specimens) with the primary rabbit antiserum or normal rabbit serum diluted 1:100 in 1% bovine serum albumin (BSA) in PBS (BSA-PBS). In formaldehyde-fixed specimens, a longer incubation time with the primary antibody was used to improve the penetration.
3. Treatment with swine serum for 40 min and rinsing in PBS.
4. Incubation for 1 h in biotinylated affinity-purified swine

immunoglobulins to rabbit immunoglobulins (Dakopatts) diluted 1:300 in BSA-PBS.

5. Treatment with swine serum for 5 min and rinsing in PBS.
6. Incubation for 30 min in a 1:600 dilution of peroxidase-conjugated streptavidin (Dakopatts) in PBS.
7. Incubation for 3 min in 3,3,'-diaminobenzidine tetrahydrochloride (DAB; Fluka, Buchs, Switzerland) (9 mg DAB in 15 ml PBS plus 10 µl 30% H_2O_2).

The sections were washed in PBS three times for 10 min after incubation steps 2, 4 and 6. Long treatments with swine serum and washings effectively prevented the non-specific staining.

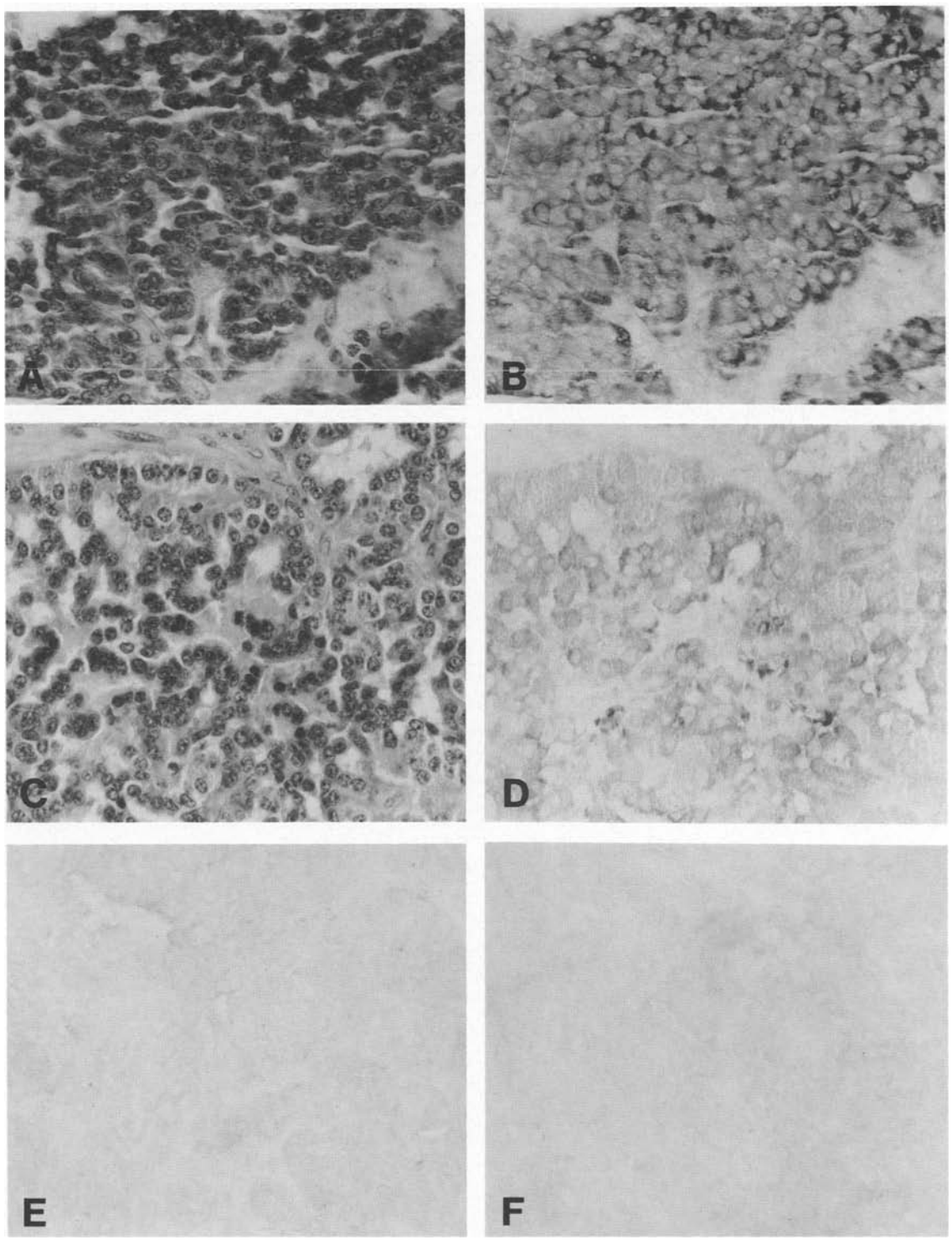

Fig. 2. Glucagonoma (A–E) and insulinoma (F) samples stained with Haematoxylin and Eosin (A, C), anti-glucagon (B), anti-CA I (D, F) and anti-CA II (E) sera. The tumour cells resemble normal islet cells and tend to form islet-like structures in the Haematoxylin and Eosin counterstained sections. The glucagonoma sample shows distinct staining for CA I (D) while the immunostaining with anti-CA II serum is negative (E). The insulinoma sample remained unstained with anti-CA I serum (F). Formaldehyde fixation. ×360.

All incubations and washings were carried out at room temperature, and the sections were finally mounted in Permount (Fisher Scientific, Fair Lawn, NJ). The sections were viewed with a Leitz Aristoplan microscope (Leitz, Wetzlar, Germany) and photographed on black and white negative film (Agfapan 25 ISO). Normal rabbit serum was used as a control serum.

Results and discussion

Carbonic anhydrase isoenzyme II is expressed in the pancreas only in the duct cells (Kumpulainen & Jalovaara, 1981; Parkkila *et al.*, 1994), from which most pancreatic cancers originate (Hootman & de Ondarza, 1993). It is, therefore, of particular interest to study whether its expression is continued in neoplastic pancreatic tissue. The present tissue samples were obtained from patients suffering from pancreatic cancer, and control samples from a patient with severe traumatic laceration of the pancreas. The histopathological examination revealed that the pancreatic tumour samples represented well-differentiated ($n = 4$) (Fig. 1, A–D, I), moderately differentiated ($n = 1$) (Fig. 1, E–H, J) and poorly differentiated ($n = 4$) ductal adenocarcinomas, cystadenocarcinoma ($n = 2$), adenosquamous carcinoma ($n = 1$), acinar adenocarcinoma ($n = 1$), and islet-cell tumour ($n = 7$) (Fig. 2). The islet-cell tumour cells histologically resembled normal endocrine cells that tended to form islet-like structures (Fig. 2, A, C). The origin of the islet-cell tumours was confirmed by subjecting the sections to immunostaining with antiglucagon, antigastrin and antiinsulin antibodies. The tissue samples obtained from the traumatic laceration of the pancreas were histologically normal and could be considered valid for reference purposes.

The paraffin sections of the samples were stained not only for CA I and II but also for CA VI in order to elucidate whether the neoplastic cells start to express other CA isoenzymes. As shown earlier (Kumpulainen & Jalovaara, 1981; Parkkila *et al.*, 1994), anti-CA II serum revealed intense staining of the epithelial duct cells of the normal tissue, while the acinar cells and endocrine Langerhans islets remained unstained (data not shown). Anti-CA I serum showed, in turn, distinct positive staining only in the peripheral Langerhans islet cells representing the glucagon-positive cells, and anti-CA VI serum showed no specific staining.

The expression of CA II in the different tumour samples is presented in Table 1. Anti-CA II serum showed from weak to intense staining in the neoplastic ductal epithelium of all adenocarcinoma samples studied (Fig. 1, B, D, F, H), suggesting that the expression of the enzyme continues during carcinogenesis. The CA II-positive staining was mainly cytoplasmic but some nuclei were also stained (Fig. 1H) as shown earlier e.g. in the human adrenal gland and duodenum (Parkkila *et al.*, 1993a; Parkkila *et al.*, 1994). The fibrous connective tissue and endocrine Langerhans islets remained unstained. The CA II-positive staining observed here in the neoplastic ductal epithelium contrasts with that described in one earlier report, in which no staining was seen with anti-CA II serum (Kumpulainen, 1984), but is in accordance with more recent observations that human pancreatic adenocarcinoma cell lines express a 30 kDa polypeptide which can be visualized using anti-CA II antibodies (Frazier *et al.*, 1990), and that most of the well-differentiated and moderately differentiated adenocarcinomas express CA II (Kim *et al.*, 1990). The discrepancy between the immunohistochemical reports may be due to the different staining methods, fixation or antisera employed. The cystadenocarcinoma and adenosquamous carcinoma cells originating from ductal epithelium also showed intense staining for CA II, while acinar adenocarcinoma sample showed only a faint staining (data not shown). The immunostainings of the ductal adenocarcinoma samples with anti-CA VI serum were negative (Fig. 1J).

Table 1. Expression of CA II in the pancreatic tumours

Tumours	−	+	++	+++
Well-differentiated ductal adenocarcinoma ($n = 4$)	0	0	2	2
Moderately differentiated ductal adenocarcinoma ($n = 1$)	0	0	0	1
Poorly differentiated ductal adenocarcinoma ($n = 4$)	0	1	3	0
Cystadenocarcinoma ($n = 2$)	0	0	0	2
Adenosquamous carcinoma ($n = 1$)	0	0	0	1
Acinar adenocarcinoma ($n = 1$)	0	1	0	0
Glucagonoma ($n = 1$)	1	0	0	0
Insulinoma ($n = 3$)	3	0	0	0
Gastrinoma ($n = 3$)	3	0	0	0

− = no staining; + = weak staining; ++ = moderate staining; +++ = intense staining.

The expression of CA isoenzymes was also studied in seven islet-cell tumour samples representing gastrinoma, glucagonoma (Fig. 2B) and insulinoma. Anti-CA I serum showed distinct staining in the glucagonoma cells (Fig. 2D) while no staining was observed with anti-CA II (Fig. 2E) or anti-CA VI sera (data not shown). No specific staining was seen in either the insulinoma or gastrinoma samples for CA I (Fig. 2F), II or VI (data not shown).

Our results clearly suggest that the expression of CA II continues in abundance in neoplastic duct cells of the pancreatic adenocarcinoma samples. CA II is known to be widely distributed throughout the epithelia of the gastrointestinal tract (Kumpulainen, 1979; Kumpulainen & Jalovaara, 1981; Spicer *et al.*, 1982; Kumpulainen, 1984; Lönnerholm *et al.*, 1985; Parkkila *et al.*, 1994) and, therefore, it is possible that it is expressed in cancer cells originating also from other epithelia of that tract. In the glucagonoma sample, anti-CA I serum distinctly stained the malignant cells. More samples should, however, be elucidated before final conclusions can be drawn about

whether this isoenzyme can be used as a marker protein for this extremely rare pancreatic tumour.

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