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Intermediate Filaments as Differentiation Markers of Normal Pancreas and Pancreas Cancer

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Expression of intermediate filaments (IF) is regulated during development and differentiation. The authors have studied the expression of vimentin and cytokeratins (CK) 4, 7, 8, 13, 18, 19 in normal pancreas, chronic pancreatitis, and pancreas cancer using monoclonal antibodies. Immunohistochemical assays were performed on fresh frozen tissue sections and on cultured pancreas cancer cells using the streptavidin-peroxidase method. In normal pancreas, acinar cells expressed CK 8 and 18, whereas ductal cells expressed CK 7, 8, 18, and 19. CK 4 was expressed by 5–10% of pancreas duct cells in all specimens of normal pancreas. CK 13 was not detected in any epithelial cells of normal pancreas or pancreatitis. CK 7, 8, 18, and 19 were homogeneously expressed in all pancreas cancers, whereas CK 4 was expressed only in 5–50% of cells in 10/16 tumors. Foci of squamous metaplasia expressed CK 13 but showed partial loss of expression of CK 7, 8, 18, and 19. Thirteen pancreas cancer cell lines examined showed homogeneous expression of CK 7, 8, 18, and 19; 2/11 lines expressed CK 4 weakly, and 6/11 expressed vimentin. CK 13 was not detected in any of the lines. These results indicate that pancreas cancer cells consistently express cytokeratin polypeptides characteristic of ductal epithelial cells and that this phenotype is retained in pancreas cancer cell lines. In addition, squamous metaplasia is associated with a coordinate change in the expression of CK polypeptides. (Am J Pathol 1992, 140:559–568)

The expression of intermediate filaments (IF) is tightly regulated during differentiation and development.[1–3] Cytokeratins (CK), the major IF proteins of epithelial cells, comprise a family of approximately 20 different related polypeptides that can be classified into two subfamilies according to biochemical and immunologic criteria:[1–4] type I CK are acidic and generally have a lower molecular weight; type II CK are neutral to basic. Two polypeptide molecules of each of the subfamilies associate to form characteristic heterotypic CK tetramers.[5,6] This phenomenon shows a certain level of specificity, i.e., CK 8 is associated with CK 18, CK 7 is associated with CK 19, and CK 13 is commonly associated with CK 4.[1,5–9] Expression of CK polypeptides is also differentially regulated during development and differentiation.[1–3,10,11] The simplest pattern of CK expression occurs in certain simple epithelia which express CK 8 and 18 polypeptides exclusively. In contrast, stratified epithelia exhibit more complex patterns of CK polypeptide expression.[1,2,12] CK expression seems to be regulated at both transcriptional and post-transcriptional levels[13,14] and current evidence suggests that type II CK polypeptides induce the transcription of genes encoding the corresponding type I CK polypeptides.[9,14] CK gene transcription in epithelial cells is regulated by enhancer elements flanking the coding sequences of CK genes or located within their intronic sequences.[9] The strict regulation of CK expression has allowed their use as histotypic markers and CK analysis is helpful in the differential diagnosis of undifferentiated tumors.[1,15–19]

In this study we have analyzed the expression of IF in normal pancreas, pancreatitis, and pancreas cancer. The results indicate that pancreas cancers consistently retain the CK phenotype of normal pancreatic ducts. In addition, some tumors showing squamous differentiation are characterized by induction of CK polypeptides normally associated with stratified epithelia. These results

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Table 1. *Characteristics of IF and the mAbs Used for Their Detection*

IF polypeptide	Mr	CK subfamily	Tissue expression	mAb	Concentration	Isotype	Reference
18	45 kD	Acidic	Simple epithelium	CK1-4	(4μg/ml)	IgG1	18
8	52.5 kD	Basic	Simple epithelium	M20	(sup1:10)*	IgG1	10, 11
19	40 kD	Acidic	Simple epithelium	LP2K	(sup1:4)	IgG2b	2
7	54 kD	Basic	Simple epithelium	CK7	(4μg/ml)	IgG1	22
13	54 kD	Acidic	Stratified epithelium	1C7	(sup1:5)	IgG2a	8
4	59 kD	Basic	Stratified epithelium	6B10	(sup1:5)	IgG1	8
Vimentin	55 kD	—	Mesenchyme	V9		IgG1	23

* sup, hybridoma culture supernatant.

provide the basis for a more exhaustive analysis of the alterations in the differentiation program of pancreas cancer cells.

Materials and Methods

Tissue Specimens

Fresh surgical specimens from 19 primary and 3 metastatic pancreas cancers were quick-frozen in isopentane cooled in liquid nitrogen and stored at −70°C. All tumors were of the duct cell type. Differentiation was graded according to the criteria of the classification of Cubilla.[20] Included were well-differentiated (n = 5), moderate-to-well differentiated (n = 3), moderately differentiated (n = 9), moderate-to-poorly differentiated (n = 2), and poorly differentiated (n = 3) cancers. Twenty three specimens of non-neoplastic pancreas were available for this study. Four tissue specimens contained normal pancreas, chronic pancreatitis, and pancreas cancer; six tissues contained normal pancreas adjacent to pancreas cancer; five tissues contained chronic pancreatitis and pancreas cancer, and eight tissues contained normal pancreas exclusively and were generally obtained from patients who underwent partial pancreatectomy during surgery for resection of other types of tumor. Most of the tissues used in this study correspond to those used in a prior analysis of the expression of blood group and blood group-related antigens in normal pancreas and pancreas cancer.[21]

Monoclonal Antibodies

The characteristics of the monoclonal antibodies (mAb) used are described in Table 1. MAbs were used as purified antibody or tissue culture supernatant as indicated; mAbs CK 1–4[18] and CK 7[22] were purchased from Boehringer Mannheim laboratories; mAb V9[23] was purchased from Sanbio (Uden, The Netherlands); mAbLP2K[2] was provided by Dr. Brigitte Lane (Imperial Cancer Research Fund, London, United Kingdom). The production and characterization of mAbs M20,[11] 1C7,[8] and 6B10[8] have already been described. These antibodies can be purchased from Euro-Diagnostics BV, Apeldoorn, The Netherlands. Antibody concentrations used are indicated in Table 1. Titration of mAbs was performed on frozen sections of normal pancreas or other tissues expressing the appropriate CK polypeptide. Isotype-matched unrelated mouse mAbs were used as controls in each experiment.

Immunohistochemical Methods

The streptavidin-peroxidase method was used. Five micron thick sections of fresh frozen tissues were fixed with cold acetone for 10 minutes. After washing with PBS, sections were incubated for 15 minutes with 1% H_2O_2 in PBS, washed, blocked with 5% normal horse serum in PBS, and incubated with primary antibody for 1 hour at 22°C. Sections were washed three times with PBS, incubated for 30 minutes with biotinylated horse anti-mouse

Table 2. *Expression of CK in Fresh Frozen Samples of Normal Pancreas and Chronic Pancreatitis*

	CK 18	CK 8	CK 19	CK 7	CK 13	CK 4
Normal pancreas						
Acinar cells	17/17	10/10	0/21	0/17	0/13	0/15
Centroacinar cells	17/17	10/10	21/21	17/17	0/13	0/15
Ductal cells	17/17	10/10	21/21	17/17	0/13	15/15*
Islets	17/17	10/10	2/21	0/17	0/13	0/15
Chronic pancreatitis						
Acinar cells	10/10	5/5	0/8	0/8	0/7	0/7
Ductal cells	10/10	5/5	8/8	8/8	0/7	7/7*
Islets	10/10	5/5	1/8	0/8	0/7	0/7

* Heterogenous staining of 10–50% of ductal cells.

Ig (1:200) (Vector Laboratories, Burlingham, CA), washed again, and incubated for 15 minutes with streptavidin-peroxidase (5 μg/ml) (Zymed Laboratories, San Francisco, CA). After washing, reactions were developed with diaminobenzidine (DAB) in PBS/H_2O_2. Sections were then counterstained with hematoxylin, dehydrated, and mounted. Percentage of reactive cells was expressed as an estimate of the whole section when staining was not homogeneous. Tumors were considered unreactive when less than 5% of the tumor cells showed DAB staining.

Cell Culture

Pancreas cancer cell lines MIA-PaCa-2, RWP-1, BxPC-3 and HS766T were obtained from the American Type Culture Collection; Capan-1, Capan-2, SK-PC-1, and SK-PC-3 were obtained from the cell line bank of the Human Cancer Immunology Laboratory (Sloan-Kettering Institute, New York, USA). IMIM-PC-1 and IMIM-PC-2 have recently been established at the Institut Municipal d'Investigació Mèdica (Barcelona). MZ-PC-1 and MZ-PC-2 were provided by Dr. Alexander Knuth (Johannes Gutenberg Universität, Mainz, Federal Republic of Germany). The cell lines that have been established recently are subject of a separate report. Cultured cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, nonessential amino acids, glutamine, penicillin, and streptomycin. Cells were plated in 60 well plates and fixed with cold methanol/acetone (1/1). Immunocytochemical assays were performed using the streptavidin-peroxidase method as described earlier.

Western Blotting

Cultured cells were scraped from flasks and washed with cold PBS. Cell pellets were lysed in 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P 40, aprotinin (50 μg/ml), 2 mM phenyl methyl sulphonyl fluoride for 30 minutes at 4°C. The insoluble material obtained by centrifugation for 30 minutes at 13,000 rpm was further solubilized in 9.5 M urea/5% 2-mercaptoethanol/2% Nonidet P-40. The urea-soluble material was used for the identification of intermediate filaments in Western blots. Proteins were separated in 9% SDS-PAGE gels and transferred to nitrocellulose filters as described, with some modifications.[24] Filters were incubated with mAbs for 1 hour at 22°C, and washed. Reactions were developed using biotinylated horse anti-mouse Ig, streptavidin-peroxidase, and DAB, at the same concentrations that were used in the immunohistochemical assays.

Results

Expression of Intermediate Filaments in Normal Pancreas

Table 2 summarizes the expression of intermediate filaments in normal pancreas. No difference was found in the pattern of IF expression in normal pancreas adjacent

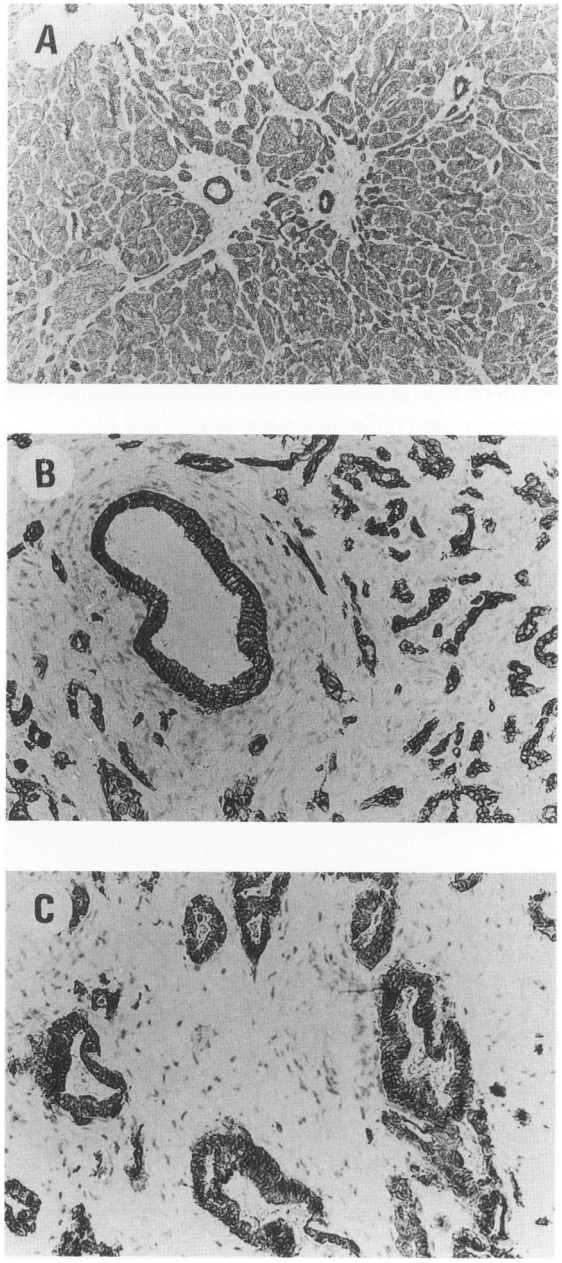

Figure 1. *Expression of CK 18 and 8 polypeptides in normal pancreas and pancreas cancer;* **(A)** *normal pancreas;* **(B, C)**, *pancreas cancer;* **(A, B)** *CK 18;* **(C)** *CK 8. Acinar, centroacinar, and ductal cells strongly express CK 18 and 8 polypeptides. Pancreas cancer cells express CK 8 and 18 homogeneously. Sections are counterstained with hematoxylin. Original magnification, ×100* **(A)**; *×200* **(B, C)**.

to pancreas cancer compared with normal pancreas from individuals without pancreas cancer. In all tissues examined, acinar cells expressed CK polypeptides 8 and 18 homogeneously (Figure 1, Table 2). CK polypeptides 7 and 19 —also characteristic of simple epithelia — were not expressed in acinar cells (Figure 2, Table 2). CK 4 and 13 — characteristic of stratified epithelia — were not expressed in acinar cells (Figure 3, Table 2). Centroacinar cells and ductal cells showed a similar CK pattern; CK 7, 8, 18, and 19 were consistently and homogeneously expressed (Figures 1, 2, Table 2). CK 13 polypeptide could not be detected in any cells of normal pancreas. CK 4 was detected in a subpopulation (10–50%) of normal ductal cells but was not detected in centroacinar cells (Figure 3). Expression of CK 4 did not correlate with duct size. No specific cytologic characteristic could be ascribed to CK 4-expressing cells in normal ducts. Islet cells consistently expressed CK 8 and CK 18, but intensity of staining was weak compared with exocrine pancreas. CK 4, 7, 13, and 19 were not detected in islet cells. Vimentin was expressed by mesenchymal cells of normal pancreas but was undetectable in all cells of exocrine and endocrine pancreas (Figure 4).

Expression of Intermediate Filaments in Chronic Pancreatitis

Table 2 summarizes the pattern of CK expression in areas of pancreatitis adjacent to pancreas cancer. No difference was noted when the expression of IF in chronic pancreatitis was compared with that of normal pancreas.

Expression of Intermediate Filaments in Pancreas Cancer

Table 3 summarizes the reactivity of mAbs detecting IF with pancreas cancer samples. There was no difference in IF expression between primary tumors and metastases. CK polypeptides 8, 18, and 19 were homogeneously expressed in all samples of pancreas cancer examined (Figures 1, 2), regardless of the degree of differentiation of the tumor. CK 7 expression was heterogeneous in 5/18 tumors and homogeneous in 13 tumors and did not relate to the degree of differentiation. CK 13 was not detected in 12/17 pancreas cancers. However, less than 5% of tumor cells expressed CK 13 in two cases

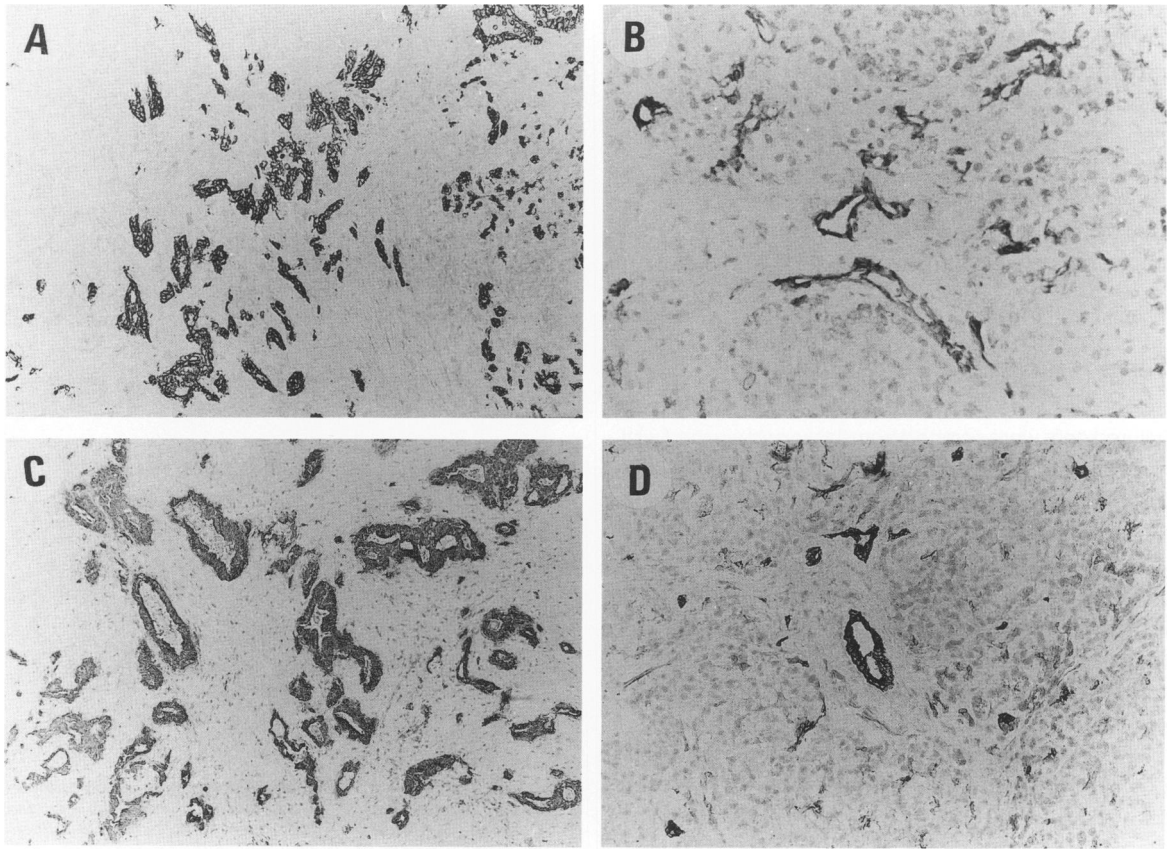

Figure 2. *Expression of CK 7 and 19 in normal pancreas and pancreas cancer;* **(A, C)** *pancreas cancer;* **(B, D)** *normal pancreas.* **(A, B)** *CK 7;* **(C, D)** *CK 19. Centroacinar and ductal cells strongly express CK 7 and 19. Pancreas cancer cells express CK 7 and 19 homogeneously. Sections are counterstained with hematoxylin. Original magnification, ×100* **(A, C);** *×200* **(B, D).**

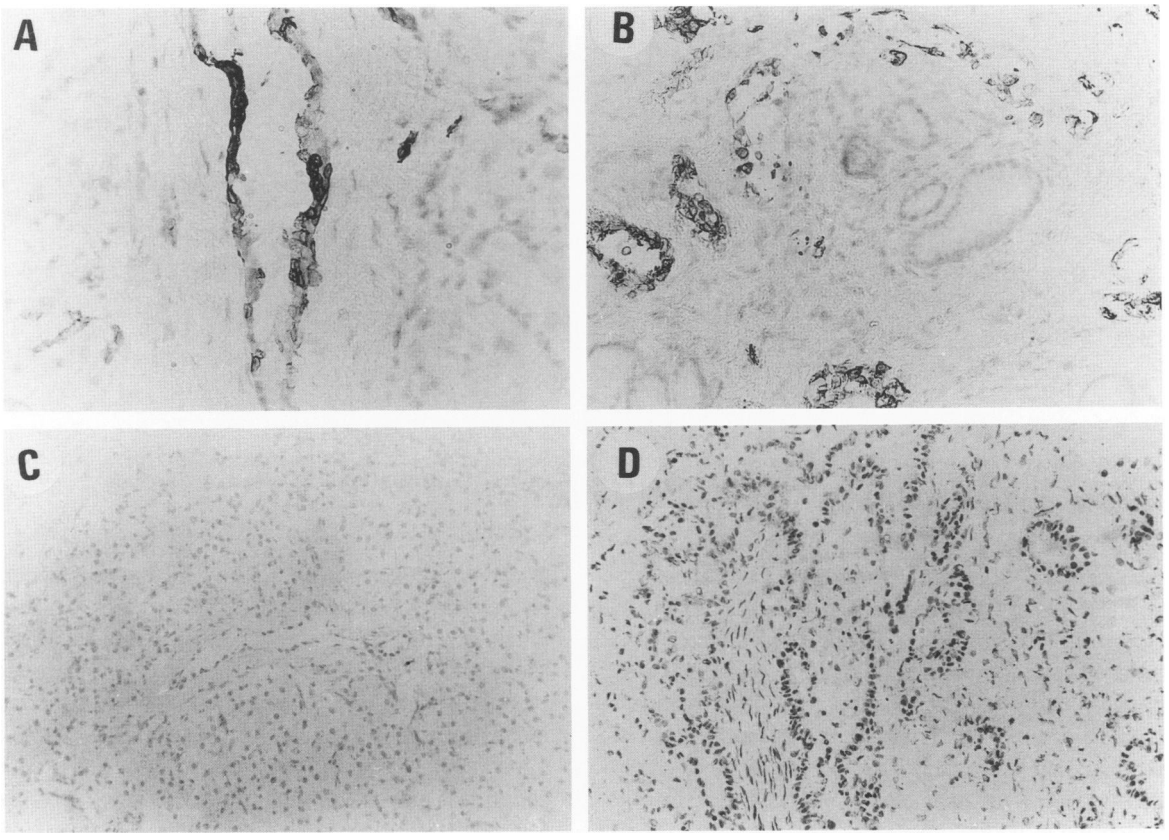

Figure 3. *Expression of CK 4 and 13 in normal pancreas and pancreas cancer;* **(A, C)** *normal pancreas;* **(B, D)** *pancreas cancer.* **(A, B)** *CK 4;* **(C, D)** *CK 13. CK 4 is expressed in a subpopulation of ductal epithelial cells whereas CK 13 is not expressed in any cells in normal pancreas. CK 4 is expressed heterogeneously in pancreas cancers showing adenocarcinoma differentiation and CK 13 is not expressed at all. Sections are counterstained with hematoxylin. Original magnification, ×400* **(A)**; *×200* **(B–D)**.

and 5–20% of cells in three tumors expressed CK 13; of the latter, one was a poorly differentiated tumor; the other two were moderately differentiated adenocarcinomas that contained areas of squamous metaplasia. Areas showing adenocarcinoma differentiation consistently lacked CK 13 expression (Figure 3), whereas a proportion of cells in areas of squamous metaplasia expressed CK 13 (Figure 5). In these tumors, the pattern of expression of CK 7, 8, 18, and 19 in areas of adenocarcinoma was identical to the pattern observed in tumors that did not contain squamous metaplasia. In areas of squamous differentiation, expression of CK 13 was generally accompanied by a lack of expression of CK 8 and 18 and decreased expression of CK 7 and 19 (Figure 5). These changes in CK expression in areas of squamous metaplasia indicate a coordinate change in the differentiation program of some tumor cells. Expression of CK 4 in pancreas cancer cells was heterogeneous, as in normal ducts. Six of 16 tumors were unreactive with anti-CK 4 mAb, whereas 10 tumors contained 5–50% CK 4-expressing cells (Figure 3). No specific cytologic characteristics could be ascribed to the CK 4-positive cells. As shown in Table 3, both primary tumors and metastases contained cells expressing CK 4. Pancreas cancer cells were always unreactive with anti-vimentin mAb in all tissues examined (Figure 4).

Expression of Intermediate Filaments in Pancreas Cancer Cell Lines

Table 5 summarizes the reactivity of mAbs detecting IF with cultured pancreas cancer cells. In general, the findings in cell lines corresponded well to the findings in pancreas cancer tissues, indicating the retention of some differentiated properties in the tumor-derived cultured cells. The results of immunocytochemical assays were confirmed by Western blot analysis of cell lysates (data not shown).

Discussion

The analysis of IF expression in tumors has shown that cancer cells retain the phenotype of the normal cell from which they arise.[1–3,15–19] In tumors derived from stratified

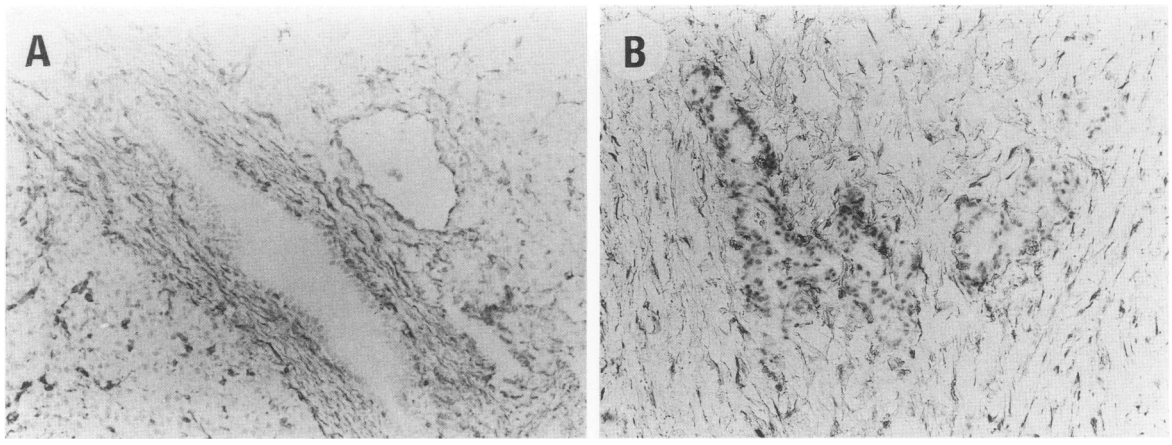

Figure 4. *Expression of vimentin in normal pancreas* **(A)** *and pancreas cancer* **(B).** *Normal acinar, centroacinar, and ductal cells do not express vimentin. Mesenchymal cells of normal pancreas are vimentin-positive. Pancreas cancer cells lack vimentin expression. Sections are counterstained with hematoxylin. Original magnification, ×200* **(A, B).**

epithelia, such as the epidermis, the tumor cell phenotype also reflects differentiation-related changes in CK expression.[8,11,12] Therefore, IF expression is often of help in determining the tissue of origin of a given tumor although it does not distinguish normal from malignant cells. To obtain further insight into the cell of origin of exocrine pancreas cancer, and to analyze the differentiated phenotype of these tumors, we have examined IF expression in normal pancreas, pancreatitis, and pancreas cancers.

In agreement with other reports, our results indicate that acinar and ductal cells of normal pancreas express different sets of CK polypeptides.[1,10,25] Acinar cells showed a simple pattern consisting of CK 8 and 18, whereas ductal cells expressed CK 7, 8, 18, and 19. CK expression in pancreas cancers was remarkably consistent and included CK 7, 8, 18, and 19, as normal ductal cells. These findings indicate that the differentiation phenotype of pancreas cancer cells is reminiscent of ductal cells, suggesting that pancreas cancers originate from normal ductal cells.

CK 4 was detected in a subpopulation of normal cells in interlobular and interlobar ducts. Previous studies have shown that CK 4 is expressed in a subpopulation of ductal epithelial cells in sweat glands, in intermediate cells of the esophageal and anal epithelia, in suprabasal cells of the transitional bladder epithelium, and in the ciliated bronchial epithelium.[1,8] Focal expression in pancreas and sweat glands ducts, and in prostatic acini, has also been reported.[8] In pancreas cancers, a variable proportion of cells expressed CK 4 in 10/16 tissues, in contrast with the homogeneous expression of CK 7, 8, 18, and 19. CK 4 expression could not be related to any specific cytologic features of the tumor cells, including squamous metaplasia. Based on these results, it appears that CK 4 distinguishes two populations of ductal cells or two stages of cellular differentiation. This question can not ultimately be answered until the differentiation of pancreas cancer cells or cells derived from normal ducts is examined *in vitro*.

CK 13, a CK polypeptide characteristic of stratified epithelia, was not detected in any cells in normal pancreas. In normal tissues, CK 13 is expressed in the suprabasal cells of the epidermis, esophagus, anal canal, and bladder, but it is absent from glandular epithelia.[1,8] CK 13 is generally present in tumors derived from internal

Table 3. *Expression of CK in Fresh Frozen Samples of Pancreas Cancer*

Pancreas cancer	CK 18	CK 8	CK 19	CK 7	CK 13	CK 4
Primary						
0–5%*	—	—	—	—	14/17	6/14
5–20%	—	—	—	—	3/17†	6/14
20–50%	—	—	—	—	—	2/14
50–90%	—	—	—	5/18	—	—
90–100%	18/18	17/17	15/15	13/18	—	—
Metastases‡	3/3	3/3	3/3	3/3	0/2	2/2

* Percentage of reactive cells. Reactivity with less than 5% of cells was considered negative.

† The three cases reactive with anti-CK13 mAb correspond to one poorly differentiated pancreas cancer and two pancreas cancers containing squamous metaplasia.

‡ One pancreas cancer metastatic to the small bowel wall, one pancreas cancer metastatic to the colon, and one pancreas cancer metastatic to the abdominal wall.

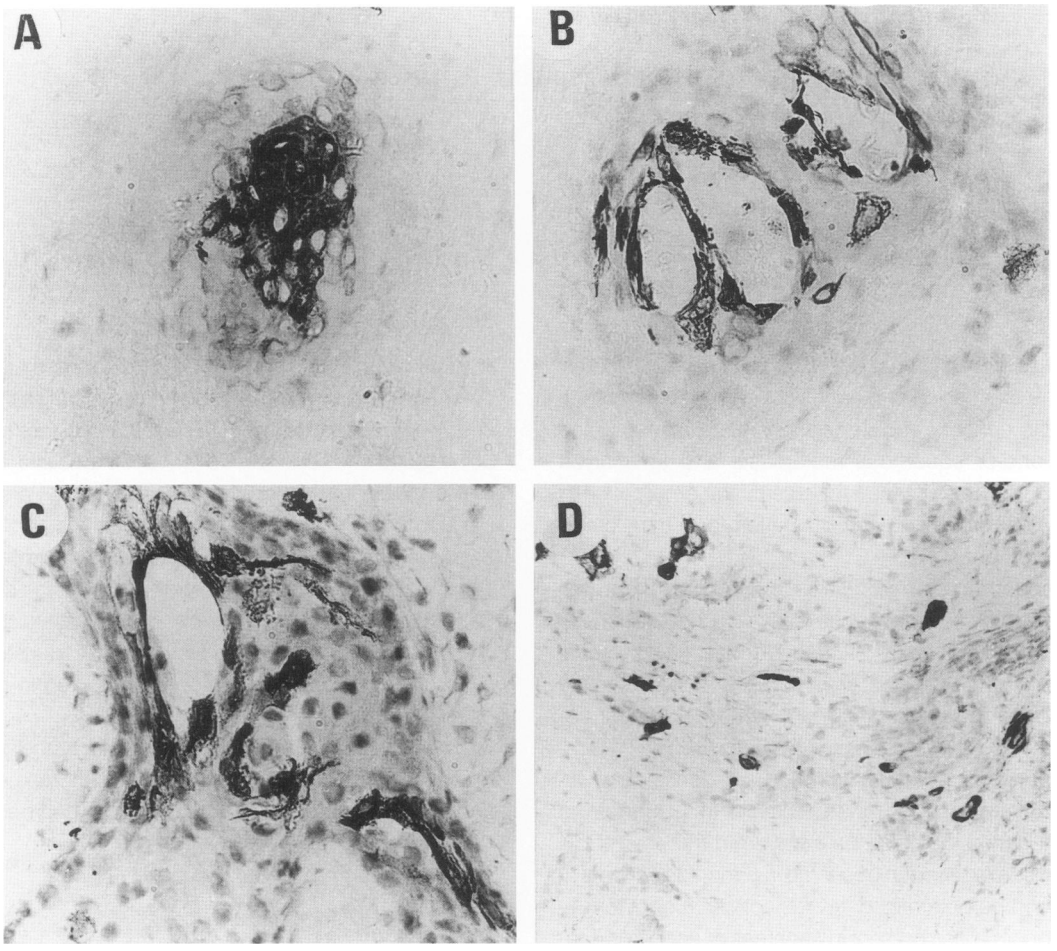

Figure 5. *Expression of CK polypeptides in areas of squamous metaplasia in pancreas cancer;* **(A)** *CK 13;* **(B)** *CK 4;* **(C)** *CK 19;* **(D)** *CK 7. CK 13 expression is induced in areas of squamous metaplasia and is associated with partial extinction of expression of CK 19 and 7. Sections are counterstained with hematoxylin. Original magnification,* ×*400* **(A–C)**; ×*200* **(D)**.

stratified epithelia.[1,8,12,16,19] Expression of CK 13 was also observed in a small subset of pancreas cancers, associated with a poorly differentiated phenotype or with squamous metaplasia. This aberrant expression of CK 13 was accompanied by a downregulation of CK characteristic of simple epithelia and may reflect a coordinate change in the expression of these differentiation markers. In another study, Kuruc et al[12] have reported CK 13 expression in areas of squamous metaplasia associated with other tumor types.

Expression of other CK polypeptides associated with cells of stratified epithelia has also been reported in a small number of pancreas cancer samples analyzed in two studies. Moll et al have described CK 17 expression in one sample of pancreas cancer using silver nitrate and Coomassie blue stains of IF preparations.[1] Using immunohistochemical assays, CK 5, which is also predominantly expressed in stratified epithelia, was detected in a subset of ductal cells in normal pancreas and in 4/14 pancreas cancers.[26] Altogether, these findings suggest that aberrant expression of CK polypeptides may be a frequent finding in pancreas cancers and may reflect an

Table 4. *CK Patterns in Normal Pancreas and Pancreas Cancer*

	CK 18	CK 8	CK 19	CK 7	CK 13	CK 4
Acinar cells	+	+	−	−	−	−
Centroacinar cells	+	+	+	+	−	−
Ductal cells	+	+	+	+	−	+/−
Islets	+	+	+/−	−	−	−
Pancreas cancer	+	+	+	+	−	+/−
Squamous metaplasia	+/−	+/−	+	+/−	+	+/−

Table 5. *Expression of IF in Pancreas Cancer Cell Lines*

IF MIA	PaCa-2	RWP1	RWP2	CAPAN-1	CAPAN-2	BxPC-3	HS766T	SK-PC-1	SK-PC-3	IMIM-PC-1	IMIM-PC-2	MZ-PC-1	MZ-PC-2
CK 18	ND*	+	+	+	+	+	+	+	+	+	+	+	+
CK 8	+	+	+	+	+	+	+	+	+	+	+	+	+
CK 19	+	+	+	+	+	+	+	+	+	+	+	+	+
CK 7	−	+	+	+	+	+	ND	+	−	+	+	+	+
CK 4	−	−	−	−	−	+	−	+/−	ND	ND	−	−	−
CK 13	−	−	−	−	−	−	−	−	ND	ND	−	−	−
Vimentin	+	+	−	−	+	−	−	−	ND	ND	+	+/−	+

* Not done.

altered differentiation pattern. It will be important to determine the expression of other CK of complex epithelia in pancreas cancers and its relationship to differentiation grade and/or squamous metaplasia and to analyze whether this phenomenon represents the emergence in tumors of a developmental CK pattern or a transformation-related phenotype.

Changes in CK expression during malignant cell transformation and oncogene activation have been reported in other studies[27–29] (and Quintanilla M, personal communication[5]). In pancreas cancers, mutations in the first exon of the K-ras proto-oncogen are the most frequent molecular alteration thus far described.[30,31] Keratinocytes transformed by activated ras genes show altered CK expression[29] (and Quintanilla M[5]), suggesting that ras genes may also be involved in the altered CK polypeptide expression observed in pancreas cancers. It remains to be determined if K-ras activation or other genetic abnormalities present in pancreas cancer cells contribute to the abnormal differentiation program observed in some tumor tissues.

Vimentin, the major IF protein of mesenchymal cells, is generally absent from normal epithelial cells but its expression may be regulated during neoplastic transformation[23,32,33] and during *in vitro* culture.[32,34] Expression of vimentin in a small subset of breast cancers has been associated with high levels of EGF receptor, low levels of estrogen receptors, and high growth fraction, indicating that it may be an indicator of aggressive biological behavior.[32,35] In our study, expression of vimentin was not detected in pancreas cancers.

Pancreas cancer cell lines showed the same CK pattern than pancreas cancer tissues. Vimentin was detected in 6/11 pancreas cancer cell lines and its expression was probably related to induction in culture. These findings indicate that pancreas cancer cell lines retain phenotypic characteristics of pancreas cancer tissues.

Misclassification of pancreas cancers appears to be a frequent problem even when histologically confirmed cases are selected for analysis[36] (and N. Malats and M. Porta, personal communication). Therefore, the identification of the common CK pattern of pancreas cancer described here and in smaller series of cases described in earlier studies[15,16,37] may be of diagnostic use. CK 7-negative tumors are unlikely to be derived from the exocrine pancreas, independent of whether they are primary or metastatic lesions. However, CK 7-positive tumors may originate from other sites in addition to the pancreas (i.e., lung, ovary, bladder, breast, bile duct).[1,15–19,37,38] Since both bile duct and pancreas cancers express CK 7,[37,38] the distinction of these two tumors on the basis of CK 7 expression is not possible. In this regard, the expression of CK 4 or CK 13 may be of diagnostic help although this question needs to be analyzed in greater detail.

The results presented provide clues for further studies regarding the differential diagnosis of pancreas cancer and the differentiation program of this tumor type.

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