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PROLIFERATION OF PARENCHYMAL EPITHELIAL CELLS ENHANCED IN CHRONIC PANCREATITIS

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SUMMARY

This study was performed to determine whether pancreatic parenchymal epithelial cells in human chronic pancreatitis tissues retain a biologically significant capability to proliferate and, if so, within which epithelial compartment proliferation occurs. The techniques of immediate per-operative *in vitro* labelling with bromodeoxyuridine (BrdU) and conventional immunohistochemistry for Ki-67 antigen expression were used to identify proliferating cells. Concordance between the two techniques was confirmed in all tissues examined. In normal pancreas, proliferation was restricted to acinar epithelial cells, with no activity in the ductules. In chronic pancreatitis of both chronic obstructive and chronic calcifying types, the number of proliferating cells in the acini was significantly increased. A small population of proliferating cells was also apparent within ductules in chronic calcifying pancreatitis, but not in chronic obstructive pancreatitis. This investigation has shown that loss of parenchymal epithelium occurring in chronic pancreatitis is not caused by a primary failure of pancreatic 'stem-cell' proliferation, but is due to disproportionate attrition of differentiated parenchymal epithelial cells by a mechanism, possibly stromal in origin, which remains hitherto unidentified. The presence of proliferating ductular cells in chronic calcifying pancreatitis, but not chronic obstructive pancreatitis, suggests that distinct pathogenic processes may be operating in the former condition, which is classically regarded as secondary to ductal obstruction by stones, and in this single respect might be considered to be identical to chronic obstructive pancreatitis. Preservation of 'stem-cell' function supports the belief that regeneration of pancreatic parenchymal tissue could be a feasible proposition if biologically appropriate management strategies were developed to treat chronic calcifying pancreatitis. © **1998 John Wiley & Sons, Ltd.**

KEY WORDS—**chronic pancreatitis; stem cells; proliferation**

INTRODUCTION

Chronic pancreatitis is an inexorably progressive disease defined as 'the presence of chronic inflammatory lesions initially characterized by selective eradication of the exocrine parenchyma, together with fibrosis, but ultimately including destruction of the endocrine parenchyma'.[1] In the U.K., approximately 1500 new cases are reported each year,[2] with the incidence rising over the past 20 years.[3] Two major aetiological categories, 'chronic obstructive pancreatitis' and 'chronic calcifying pancreatitis', are recognized.[4] The first is due to proximal obstruction of duct drainage by a variety of neoplastic and non-neoplastic lesions. The second encompasses several subtypes, all believed to arise as a result of intraductal stone formation, although the aetiopathogenesis remains elusive. In Western countries, alcohol abuse,[5] genetic predisposition,[6] diets deficient in micronutrients and protein, together with abnormal handling of xenobiotics, smoking, and drug toxicity have all been proposed as aetiological agents.[5–8] Autoimmune rejection of the pancreas has also been postulated.[9] Between 9 and 41 per cent of cases remain idiopathic.[2] Nevertheless, the precise aetiopathological factors contributing to the progressive loss of parenchymal elements, and their replacement by dense fibrous connective tissue, have not yet been incontrovertibly identified.[10]

The pancreatic parenchyma is believed to exhibit continuous but conditional renewal. In rodents, complete regeneration follows partial pancreatic resection, whereas in the human, there is only limited evidence of compensatory regeneration following loss of pancreatic tissue.[11] Depletion of the exocrine epithelial component characterizes all types of chronic pancreatitis, although it is not known whether this is due to excess destruction of fully-differentiated epithelial cells, including those with residual proliferative capacity, or a failure of pancreatic stem cells to divide and to repopulate the organ.

The present study was performed to determine whether a biologically significant proliferative capacity might be demonstrated in pancreatic tissues affected by both forms of chronic pancreatitis. Two independent markers of cell cycle parameters have been examined: bromodeoxyuridine (BrdU), a dynamic marker of proliferation[12] incorporated into the cell cycle during S-phase of cell division, was detected immunohistochemically following intraoperative incubation of pancreatic tissue samples. Expression of Ki-67, a

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proliferation-association protein tightly bound by chromatin in all cells as they progress from S into G_2 phase of the cell cycle, was also detected immunohistochemically.[13] The findings of this study have confirmed that pancreatic epithelia in chronic pancreatitis maintain an enhanced proliferative capacity, which has yet to be exploited in the management of patients with this group of diseases.

MATERIALS AND METHODS

Case material

Fresh pancreatic specimens were collected from 12 partial pancreatectomy cases performed between December 1993 and September 1994 at the Hammersmith Hospital, London. Of these 12, four comprised control specimens obtained from patients undergoing resection for non-obstructing, small neuroendocrine tumours and benign cystic lesions. Pathological tissues were obtained from four cases of chronic obstructive pancreatitis and four cases of chronic calcifying pancreatitis, the latter being of probable alcoholic origin. The total complement of tissues examined comprised five females and seven males, with a mean age of 58 years and an age range of 24–74 years.[10,14]

Tissue collection

Tissues were collected intraoperatively and three representative slices were taken from each at least 1 cm from the surgical resection margin, according to the protocol described in our previous publications.[10,14] Each piece of tissue was diced into several cubes (1–3 mm diameter) and incubated in air-buffered Leibowtiz medium, pre-warmed to 37°C.

BrdU incorporation technique

Tissue samples were processed within 30 min according to the BrdU *in vitro* techniques.[15] For each specimen, three incubation chambers were set up. In each chamber, three pieces of pancreatic tissue, each approximately 1·5 mm^3, were incubated in 1·5 ml of HEPES-buffered Leibovtiz medium containing 0·15 per cent (w/v) BrdU under hyperbaric conditions, and the tubes closed with perforated stoppers. Incubation was carried out for 1 h at 37°C. Thereafter, the screw of the apparatus was gently released under water to allow excess oxygen to escape. Tissues were then processed individually into paraffin wax.

Tissue preparation

Paraffin sections—Sections cut at 5 μm thickness were mounted onto poly-L-lysine-coated slides for the BrdU sections and onto Superfrost slides to improve adhesion during microwaving for the Ki-67-stained sections. After drying overnight, sections were dewaxed in xylene and rehydrated using graded alcohols. Consecutive sections were used for the two investigative procedures.

Immunohistochemistry

Control tissues—Sections of rat liver from animals flash-labelled with BrdU (50 mg/kg body weight) for 1 h prior to killing were used as controls for the *in vitro* BrdU-labelled human tissues. Sections of human small intestine were used as controls for Ki-67 immunohistochemical staining.

Pretreatment requirements prior to immunohistochemistry

Trypsinizationlhydrochloric acid pretreatment of BrdU sections—After blocking endogenous peroxidase by immersion in 0·1 per cent hydrogen peroxide in methanol for 30 min at room temperature, sections were washed in pre-warmed 37°C distilled water; pre-digested in freshly prepared trypsin (Sigma, U.K.) solution, 1 mg/ml, in calcium chloride solution (0·66 g/500 ml), pH 7·8, for 13 min at 37°C; rewashed and heated in normal hydrochloric acid, for 15 min, at 60°C to improve nuclear penetration; and finally washed thoroughly in distilled water and Tris-buffered saline (TBS, pH 7·6) before immunohistochemistry.

Microwave pretreatment of Ki-67 sections—The Ki-67 sections were microwave-treated at 700 W for 10 min and immersed in pre-warmed 10 mmol citrate buffer solution, pH 6·0 (2·1 g of citric acid in 1·0 l of distilled water). Endogenous peroxidase was then blocked using 0·3 per cent H_2O_2 in water for 30 min before rinsing in phosphate-buffered saline (PBS, pH 7·4).

Immunohistochemical technique

The BrdU sections were washed throughout in TBS and the Ki-67 sections in PBS. Non-specific protein binding was inhibited with normal rabbit serum at a dilution of 1:20. Sections were incubated with primary antibody in a moist chamber, at room temperature, for 2 h. The anti-BrdU (Dako, Denmark) antibody was diluted to 1:30 in 1 per cent human A-B serum in TBS and the anti-Ki-67 antibody (Dako, Denmark) was diluted to 1:50 in PBS. As a negative control, the primary antibody was replaced by buffer. BrdU-labelled sections were then washed and incubated with biotinylated rabbit anti-mouse immunoglobulin (Dako, Denmark; diluted 1:300 in 1 per cent human serum in PBS) for 1 h. After washing, sections were incubated with ABC complex (Dako, Denmark) for 45 min. Anti-Ki-67 antibody-labelled sections were incubated in swine (anti-rabbit) immunoglobulin (Dako, Denmark) at a dilution of 1:100 in PBS for 1 h and then washed before further incubation for 1 h with rabbit peroxidase anti-peroxidase antibody (Dako, dilution 1:300 in PBS). All sections were then developed by immersion in a 0·5 per cent (v/v) solution of 3,3′-diaminobenzidine hydrochloride (DAB; Aldrich, Gillingham, U.K.) in PBS containing 0·03 per cent (v/v) H_2O_2 after final washing. Nuclei were lightly counterstained with haematoxylin before mounting.

Stained sections were scored for BrdU and Ki-67 labelling by light microscopy at a magnification of × 400. Both antibodies produced stained positive nuclei. The total number of epithelial nuclei, together with positively staining nuclei, in ten randomly selected fields

Table I—Incidence of mitoses per 10^4 epithelial nuclei (acinar+ductular)

Tissue	Acini		Ductules	
	BrdU	Ki-67	BrdU	Ki-67
Normal pancreas	8·95 ± 2·6	11·8 ± 9·3	0·0	0·0
Chronic obstructive pancreatitis	27·0 ± 8·3	24·5 ± 15·1	0·0	0·0
Chronic calcifying pancreatitis	29·3 ± 21·8	33·5 ± 10·2	9·4 ± 9·0	6·8 ± 8·0

was recorded for the three specimens taken in each case. The location of positive-staining nuclei was recorded as ductal or acinar.

RESULTS

Table I shows the mean proliferation rates per 10 000 epithelial cell nuclei within each histological category (acini or ducts) for the proliferation markers BrdU and Ki-67, respectively.

In control pancreatic tissues, the proliferating compartment was restricted to acinar epithelial cells. In chronic obstructive pancreatitis, epithelial cell division was greater than in normal pancreas, although restricted to acinar cells, with no proliferation identified in ductules (Fig. 1). However, in chronic calcifying pancreatitis, not only was acinar epithelial cell division greater than that found in control tissues, but also a population of dividing cells was identified within ductular epithelium (Fig. 2). In both categories of chronic pancreatitis, acinar mitoses were more frequent at the margins than in the centres of affected lobules (Fig. 3).

In general, the Ki-67-determined proliferation rates were slightly higher than the BrdU-labelled cells, as expected, since Ki-67 tends to overestimate the growth fraction.[16] There was, however, a close and consistent correlation between the two techniques for all groups of data examined.

DISCUSSION

This study has shown that in control, non-inflamed, human pancreatic epithelium, there is a low resting rate of acinar cell division of between 0·06 and 0·11 per cent. This finding is consistent with an earlier study of human pancreatic cell division which used [^{3}H]thymidine incorporation as the proliferation marker and indicated the number of labelled cells to be extremely low (0·1–0·2 per cent).[11] That report led to the current belief that human exocrine pancreatic epithelium comprised cell populations of the slowly expanding type, with little capacity to undergo regenerative proliferation.[17] Nevertheless, it is recognized from animal model studies that, under appropriate conditions, pancreatic parenchyma is able to enter into cell division, producing hyperplasia. In human pancreatic tissue, there is some evidence of regeneration following episodes of acute pancreatitis.[11]

The present study is the first investigation to document the proliferative potential of the human pancreas in chronic pancreatitis. The data have clearly shown that cell division in the acinar epithelial compartment is enhanced in both forms of chronic pancreatitis, with a proliferation rate approximately three to four times greater than in normal (non-inflamed) pancreatic tissues. Thus, in human chronic pancreatitis, atrophy of acinar units is now confirmed as being attributable to an imbalance between increased epithelial cell loss and the readily demonstrated enhanced proliferative potential. In rat and mouse models where duct ligation, partial resection, and chemical damage have been employed to stimulate regeneration, the mitotic rates determined using *in vivo* BrdU and [^{3}H]thymidine pulse-labelling techniques were increased by three to five times.[18–20]

With respect to the microanatomical location of the proliferating cells, no mitoses were identified in the ductular epithelium of the normal pancreas, or of chronic obstructive pancreatitis tissues, but were found in chronic calcifying pancreatitis tissues. Comparison of the data from the four cases of chronic obstructive pancreatitis with the four cases of chronic calcifying pancreatitis suggests that ductule recruitment into active cell division is a unique feature of chronic calcifying pancreatitis. If this finding is representative of the two morphological groups, then it is a strong indication that the pathogenesis of chronic calcifying pancreatitis may not be simply that of duct obstruction by stones, a view substantiated by the recent findings of Ectors *et al.*[21] However, the duration of the primary insult in chronic calcifying pancreatitis may control the extent of recruitment of residual stem cells into the mitotic compartment.

The location of any putative stem cells within the pancreas remains controversial. At present, there is no convincing evidence for a single pancreatic precursor cell, for trans-differentiation, or for dedifferentiation of exocrine epithelial cells.[11] In animal models, following growth stimulation, mitotically active pancreatic epithelial cells exhibit characteristics of differentiated cells, thus indicating that each individual cell population is able to undergo restoration following previous loss. In these models, the majority of such cells appear to be of the acinar phenotype, but are unlikely to be truly terminally differentiated. However, the centroacinar cell, which lies at the centre of each acinar unit, has been postulated as the main parenchymal stem cell.[22–25] In rats, 90 per cent partial pancreatectomy is followed by substantial exocrine and endocrine regeneration, in which proliferation and differentiation of ductal epithelial cells occur.[18] In another study, a lag phase was identified between commencement of ductular cell

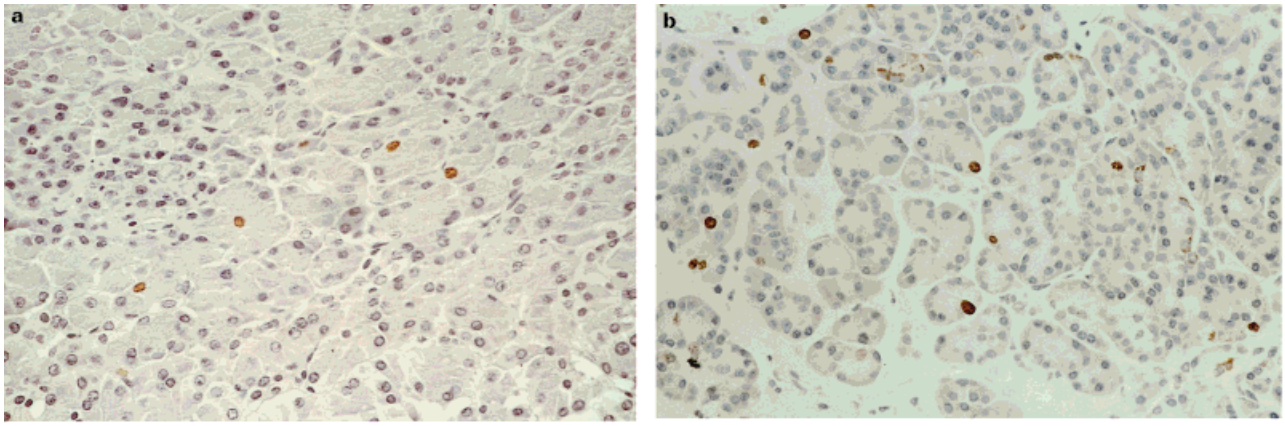

Fig. 1—(a) Early chronic obstructive pancreatitis in which pancreatic lobular architecture remains well preserved. A small collection of chronic inflammatory cells is present (left) where they are associated with focal epithelial cell drop-out. Proliferatively active cell nuclei are clearly labelled with BrdU. × 250. (b) Early chronic obstructive pancreatitis in which lobular architecture is preserved but in which intralobular fibrous connective tissue is increased. Cell proliferation within the acinar epithelial compartment is clearly demonstrated by immunohistochemical localization of Ki-67 protein. × 250

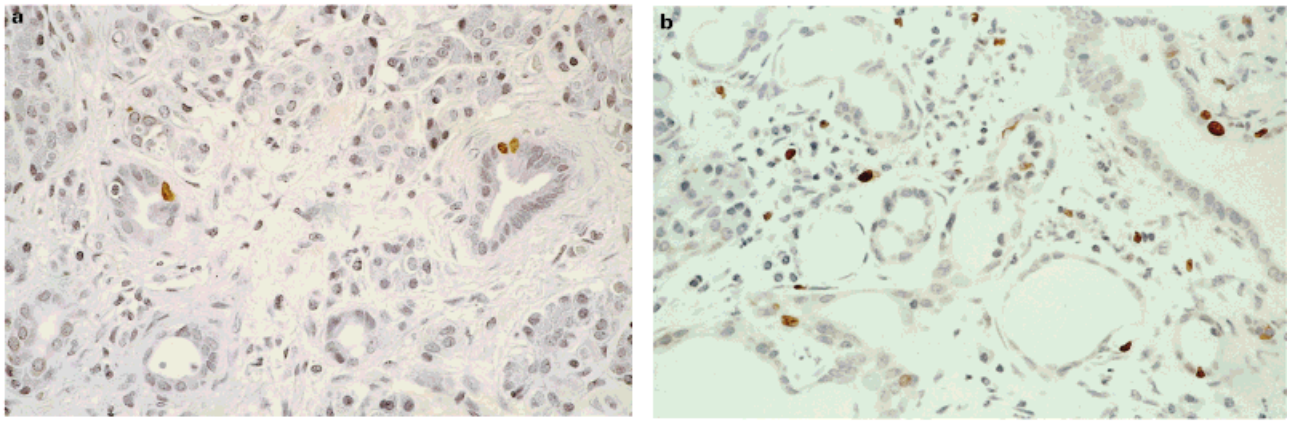

Fig. 2—(a) Established chronic calcifying pancreatitis in which nuclei in residual second-order duct epithelial cells are clearly labelled with BrdU. Within the centre of a lobular structure, no local acinar epithelial cells were similarly labelled. × 250. (b) Late-stage chronic calcifying pancreatitis in which nuclei of individual residual acinar and ductular epithelial cells both express immunohistochemically reactive Ki-67 protein. × 250

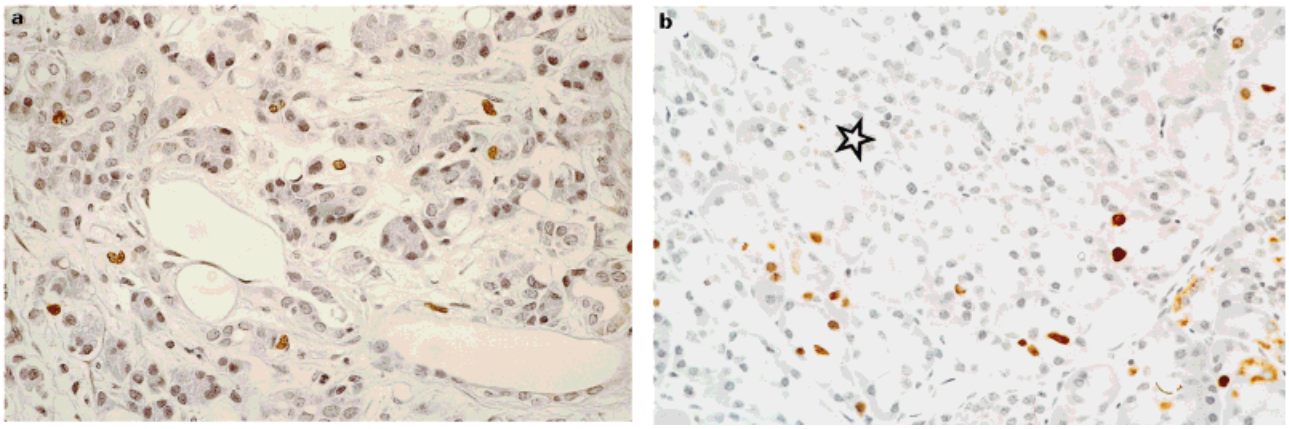

Fig. 3—(a) Periphery of lobule in chronic calcifying pancreatitis. Multiple nuclei, particularly of acinar epithelial origin, are strongly labelled with BrdU. × 250. (b) In many lobules, proliferating nuclei were particularly located around the peripheral rim of acini when compared with the relatively quiescent central acini. A star identifies the terminal ductular structure at the centre of the acinus × 250.

division and onset of acinar mitosis.[17] It was suggested that the acinar cells which divide are, most probably, those cells from the ductular compartment which have become differentiated and it was hypothesized that the pancreas is composed of an integrated series of proliferative units, each comprising acinar cells, centroacinar cells, and ductules, and reacting as an integrated unit whenever acinar cell loss occurs.[17]

In the present study, the principal site of epithelial cell proliferation was identified at the margins of lobules adjacent to the fibrous bands characteristic of chronic pancreatitis. We suggest that an interaction between residual epithelial cells retaining a proliferative capacity and the adjacent specialized pancreatic stroma may be an important factor in pancreatic regeneration. Mesenchyme is essential for normal pancreatic growth, possibly by binding growth factors to the mesenchymal proteoglycans and thus presenting these to the epithelial cells via a basement membrane.[26] Connective tissue appears to support the adult differentiated state by inhibiting the formation of acinar cells and localizing only around large ducts in the normal pancreas.[11] There may also be a role for transforming growth factor beta (TGF-β_1), which we have shown to be enhanced within the ductules in chronic pancreatitis of both types.[14] TGF-β_1 is known to promote fibrosis and to limit cell proliferation,[14] possibly acting as an autocrine regulator of proliferation.[27]

In summary, the present study has confirmed that in chronic obstructive pancreatitis, and in chronic calcifying pancreatitis, the proliferative potential of acinar cells is not only preserved but also enhanced, when compared with non-inflamed pancreatic tissues, as assessed by the independent techniques of *in vitro* BrdU labelling and expression of Ki-67 protein. Furthermore, in chronic calcifying pancreatitis, ductular epithelial cells also become recruited into the proliferating compartment. In contrast, large ductal epithelial cells do not appear to become recruited or to participate in proliferation. These data not only suggest that the pathogenesis of the two types of chronic pancreatitis is likely to be distinct, thus supporting the recent opinion expressed by Ectors *et al.*,[21] but also suggest that in both disease groups, loss of pancreatic exocrine parenchyma is not due to a primary failure of pancreatic epithelial cell proliferation but to another (possibly stromal) mechanism which has yet to be identified. Whether or not the cells derived from these proliferating epithelial compartments might recapitulate the full spectrum of parenchymal functions depends on a variety of factors, including the composition and interaction of the adjacent residual stroma. Nevertheless, despite these obvious caveats, the potential remains for developing a more biologically appropriate strategy to manage patients with chronic pancreatitis, rather than the non-selective ablative surgical approach currently employed.[28]

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