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Original article

In vitro pancreatic carcinogenesis

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Summary

Studies in our laboratories have indicated that pancreatic cancer originates not only from pancreatic ductal/ductular cells but also from within the Langerhans' islets, probably from reserve (precursor, stem) cells. To identify, enrich and characterize these cells, we established a long-term hamster islet culture and studied their growth, differentiation and response to the pancreatic carcinogen, N-nitrosobis(2-oxopropyl)amine (BOP). One group of cultured islets was treated *in vitro* with BOP (KL5B group) and the other group of islets served as an untreated control (KL5N group). During the early culture days, in both groups all cultured islets showed a progressive loss of endocrine cells and replacement by ductular, acinar and intermediary cells. However, all these cells disappeared after 35 days in culture and gave room to undifferentiated cells, which we believe represent stem cells. No differences were found between KL5N and KL5B cells with regard to cell growth and differentiation until day 35, when the growth of the KL5B cells accelerated and the cells underwent increasing pleomorphism and atypia. At day 133, KL5B cells but not KL5N cells showed colony formation in soft agar and formed invasive, poorly differentiated adenocarcinomas of the ductal type when transplanted into hamsters. All of these tumors showed mutation of the K-*ras* gene and extensive chromosomal damage. We concluded that like ductal/ductular cells, certain cell populations within islets are responsive to the carcinogenic effect of BOP. We could not ascertain whether these cells present a preexisting (stem, reserve) cell population within the islets or transdifferentiated islet cells.

Key words: carcinogenesis, culture, differentiation, hamster, islets, pancreas

Introduction

The histogenesis of pancreatic cancer is obscure. The opinion about the tumor precursor cells varies and includes ductal cells, ductular cell and acinar cells. Studies in the hamster pancreatic cancer model, which in many aspects resembles human pancreatic cancer [1-7], have indicated that tumors arise from ductal and ductular cells, as well as within islets [8]. In fact, the earliest alteration during cancer development is the formation of intrainsular ductular structures that give rise either to lesions compatible with the human microcystic adenomas, or malignant glandular structures that destroy the islets and invade the surrounding tissue even when they are microscopic in size. On the contrary, ductal lesions develop much later, and remain within the ductal boundary for a long time before they become invasive. This pattern is consistent with intraductal tumors in humans. The greater malignancy of intrainsular cancers is thought to be due to the exposure of the evolving tumor cells to a high concentration of growth factors within the islet environment, and the lack of a connective tissue barrier around the islets. Although our studies have substantiated the role of islets in pancreatic carcinogenesis [9,10], the identity of the tumor precursor cells, which we believe represent stem (reserve) cells remains obscure. To identify, characterize and enrich these stem cells, and test their response to the pancreatic carcinogen, N-nitrosobis(2-oxopropyl)amine (BOP), we established a long-term hamster islet culture.

Material and methods

Islet isolation

Pancreatic islets of female hamsters were isolated and purified as described [11].

Islet culture

Isolated islets were cultured in M3:5 medium (InCell™, San Antonio, TX) and were kept on a rocker for 14 days to prevent their attachment to the bottom of the dish and to remove fibroblasts. The islets were divided into two groups, KL5N (200 islets, no BOP treatment) and KL5B (250 islets treated with BOP). The floating islets were handpicked every day, counted and put into a new petri dish containing fresh medium to separate them from the fibroblasts that attached to the bottom of the flask. On day 15 from the initial isolation, when no fibroblast contamination was evident anymore, islets from each group were removed from the rocker, transferred into six-well tissue culture plates and allowed to attach. They were continuously cultured without shaking and subcultured in M3:5 when subconfluent. Portions of the cells were harvested for soft agar assays, K-*ras* mutation analysis, histology/immunohistochemistry, or electron microscopy.

BOP treatment

KL5B Islets were treated with 0.25mM BOP from the first day of islet culture. A pilot study had shown that this concentration had no toxic effect and was well tolerated by the islet cells. The media were changed every day for the first 14 days and three times per week thereafter. BOP was added to the fresh medium for the BOP treated group and discontinued after day 133.

Histochemical and immunocytochemical examinations

All examinations, including the multilabeling technique, were performed as previously described [12]. Antibodies used in this experiment are reported in our earlier study [11,13].

Electron microscopy

The subcellular patterns of the cells at different culture days were examined as reported earlier [14].

Anchorage-independent growth
assay was performed as reported earlier [15]. Formation of colonies was checked once a week and the assay discarded after 45 days.

Transplantation experiment
About 1 million KL5B or KL5N cells at day 133 were transplanted into the subcutaneous tissue, the submandibular gland, or into the pancreas of recipient hamsters (three hamsters per site), as reported [16]. Subcutaneous masses, lesions in the submandibular gland and pancreas were removed at autopsy, and all organs were examined grossly for metastases. The regional lymph nodes of these hamsters and the pancreas of three age-matched control hamsters were removed, fixed in 10% buffered formalin, and processed for histology by conventional methods.

Examination of the K-ras mutation
The mutation of K-*ras* was examined by RT-PCR and direct sequencing, according to a previously described method [15].

Cytogenetic analysis
The karyotype of KL5N and KL5B at days 133 and 238 was performed as reported [15]. Fifty-one KL5B and forty-two KL5N cells were examined at each sample.

Results

Islet culture

The growth patterns of both KL5N and KL5B islets were similar. Islets retained their original size and spherical shape, and showed the same distribution of individual islet cells as in the native pancreas. Examination of 500 freshly isolated cells by immunohistochemistry (using antibodies against islet hormones and hamster ductal specific pancytokeratin) and electron microscopy did not show any exocrine cells attached to the islets. Beginning at day seven, β-cells showed necrosis, their number decreased rapidly and were replaced by an increasing number of ductular structures. These ductular structures all appeared in the center of the islets (Fig. 1). Initially they formed minute conducts which could be detected electron microscopically (Figs. 2, 3). The ductules, composed of cells of various sizes, showed regular microvillae with junctional complexes between each other (Fig. 3) and were surrounded by endocrine cells. The α- and δ-cells, identified by the respective antibodies, occupied the peripheral islet region and survived about one to two weeks longer than β-cells. At day 14, in the KL5N and KL5B culture, the number of floating islets was reduced by 30%. At day 21, after one week off the rocker, single cells or small groups of acinar cells, and a few intermediary cells were found between the ductular and endocrine cells. The latter cells were still immunoreactive to anti-glucagon and anti-somatostatin but not to anti-insulin anymore. At day 28, the core of the islets was consistent of amorphous material that contained nuclear shadows (Fig. 4). The surface of the core was covered by cuboidal or cylindrical epithelial cells forming a monolayer or multilayers (Fig. 4). These cells expressed pancytokeratin but not endocrine islet cell markers. At day 35, both KL5N and KL5B presented an undifferentiated cell type with large eosinophilic cytoplasm and poor in cell organelles. A few small cells with hyperchromatic nuclei were scattered between the large cells.

At day 56, the growth rate of KL5B cells accelerated and showed a doubling time of 36 hours, compared to 48 hours in KL5N cells. Although KL5N cells retained their monomorphic phenotype (Fig. 5a), the KL5B group was composed of a mixture of normal looking cells and of small

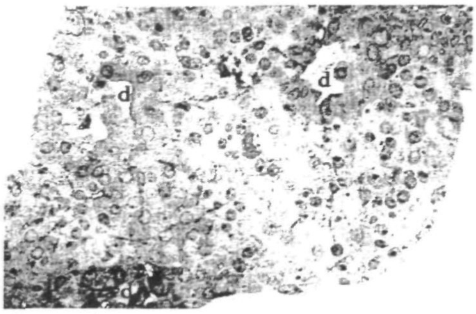

Figure 1: An islet in culture for seven days. Two small ductular structures (d) surrounded by endocrine cells are seen in the center of the islet. A few large epithelial cells (arrow) were scattered between the endocrine cells. H&E x 40.

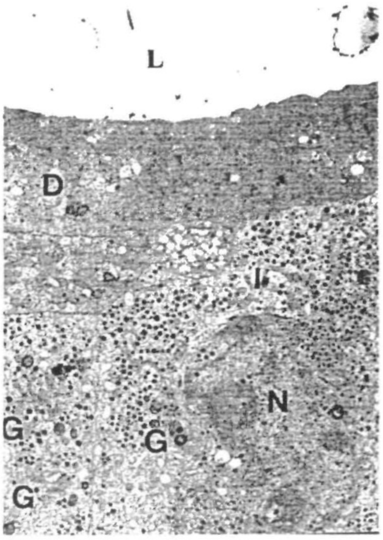

Figure 2: Ultrastructure of a similar ductule as in Fig. 1 showing a portion of ductular cells (D) between the lumen (L) and endocrine cells with granules, and Golgi stacks (G) around the nucleus (N). x 3000.

Figure 3: A minute ductule within an islet at day seven. Ductular structures formed by small epithelial cells are surrounded by endocrine islet cells. Portion of a ductular cell extends between two endocrine cells (arrows). X3000.

Figure 4: An islet at day 28. The islet core (C) presents amorphous meshy material with nuclear shadows. Accumulation of cuboidal or cylindric epithelial cells on the surface of the core. H&E x 25.

cells with hyperchromatic nuclei (Fig. 5b). The number of the small cells increased gradually and at day 133 the culture was composed entirely of small cells with pleomorphic and hyperchromatic nuclei, and scanty eosinophilic cytoplasm (Fig. 5c).

Anchorage-independent growth
At day 133, in contrary to KL5N cells, KL5B cells showed for the first time anchorage independent growth in soft agar and could be maintained in RPMI-1640 culture medium supplemented with 10% fetal bovine serum.

Activation of K-ras oncogene
The mutation of the K-*ras* was found only in KL5B cells at day 133 and later. The mutation was in codon 12 (GGT→GAT), as found in primary pancreatic cancers [1], in the cell lines derived from them, and in BOP-induced tumors arising from islets *in vivo* [17].

In vivo *growth patterns of KL5B cells*
One million KL5B cells injected into the subcutaneous tissue of all 9 hamsters grew up to 20-mm nodules within two weeks. All of these tumors were invasive, and depending on the site of tumor cell inoculation, they had invaded the abdominal wall, the peritoneum, pancreas, or the submandibular glands. No metastases were detected. Histologically, tumors of all three sites were anaplastic with massive necrosis and hemorrhage (Fig.6).

Cytogenetic analysis
Cytogenetic analysis of KL5N at day 133 showed a normal hamster chromosome complement [13]. However, forty-five KL5B cells presented an abnormal clone characterized by a missing Y, monosomy 7 and 11, one copy of two markers, and two copies of another marker. Six cells were a tetraploid version of this clone.

Immunohistochemical findings
Examination of KL5N and KL5B cells at day 133 showed that expression of laminin, EGFR and its ligand TGF-α (Fig 7) was much higher in KL5B cells than in KL5N cells (Table 1). Both KL5N and KL5B cells were about the same reactive to antibodies against pancytokeratin, cytokeratins 14, 18, carbonic anhydrase, vimentin, tomato lectin, PHA-L, and α-1-antitrypsin. Immunohistochemical findings in tumors of different transplantation sites are summarized in Table 2. We found a higher expression of EGFR and TGF-α in the tumors grown in the SMG and pancreas compared to the subcutaneous grown tissue.

Discussion

Genetic changes found in hyperplastic and dysplastic lesions of ducts in human individuals without pancreatic cancer [18], as well as in carcinoma *in situ* or pancreatic cancer, imply that ductal cells are the tumor progenitor cells. Also, in the hamster pancreatic cancer model, tumors derive from ducts and ductules [19]. And the malignant transformation of hamster ductal cells treated *in vitro* with BOP clearly points to the susceptibility of hamster ductal cells to malignancy. However, in the hamster treated with BOP, most cancers develop within islets [20] and several studies have indicated that the presence of intact islet cells is prerequisite for pancreatic cancer induction by BOP [20,21]. Destruction of islets by diabetogenic compounds inhibits or

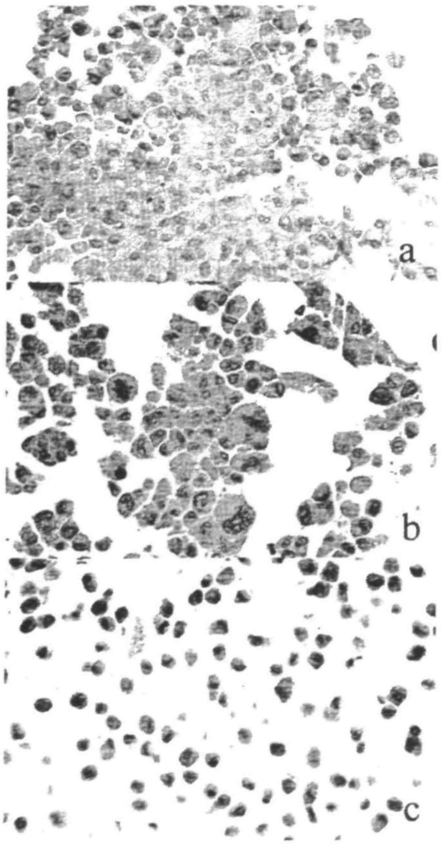

Figure 5: a) KL5N cells after 56 days in culture. Undifferentiated uniform monomorphic cells with large cytoplasm and regular nuclei with tendency to adhere to each other. H&E x210. b) KL5B cells at day 56. Pleomorphic cell population with small and large cells. H&E x210. c) KL5B cells at day 133. Small, pleomorphic cells with hyperchromatic nuclei and small cytoplasms. At this stage, the cells grew in soft agar and could be maintained in RPMI-1640.

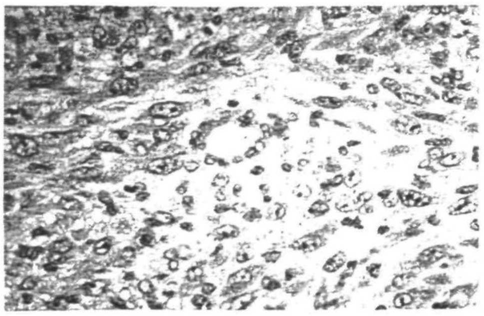

Figure 6: Histological pattern of KL5B cells obtained from a subcutaneous tumor grown in a hamster. Anaplastic tumor is composed of pleomorphic cells forming occasionally minute glandular structures. Large necrotic and hemorrhagic areas were present in the center of the tumor. H&E x210.

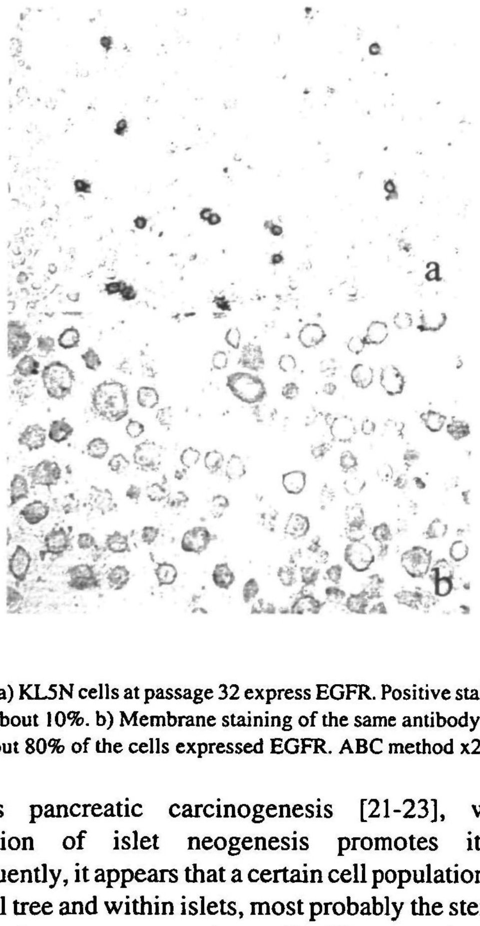

Figure 7: a) KL5N cells at passage 32 express EGFR. Positive staining was found in about 10%. b) Membrane staining of the same antibody in KL5B cells. About 80% of the cells expressed EGFR. ABC method x210.

prevents pancreatic carcinogenesis [21-23], whereas stimulation of islet neogenesis promotes it [24]. Consequently, it appears that a certain cell population within the ducal tree and within islets, most probably the stem cells, presents the tumor progenitor cells. However, it was not clear whether tumors developing within islets, derive from undifferentiated precursor (stem cells) or from transdifferentiated islet cells. To answer these questions, we established a hamster islet culture and treated the islet cells with BOP *in vitro*. The results confirmed our *in vivo* observation that, like ductal cells, some cells within islets have the potency to give rise to an exocrine cell tumor. The origin of these cells from exocrine cells attached to the islets could be excluded because in over 500 islets that were purified by our technique and examined in a pilot study, immunohistochemically and electron microscopically did not reveal any exocrine cell contamination. Moreover, contrary to freshly isolated islets, all islets in culture showed formation of many ductules as early as 7 days in culture and the appearance of these ductular structures coincided with the necrosis and disappearance of the β-cells. Our results are in line with observations by other investigators [25-27], and suggest, that human islets in culture differentiate into ductular cells. Strikingly, in our study intermediary and acinar cells were also developed within cultured islets but were not found in the primary islets, indicating a pluripotent differentiation capability of islet cells. Whether these exocrine cells derive from preexisting precursor (stem) cells or from transdifferentiated islet cells is debatable.

Nevertheless, in culture, BOP transformed hamster islets. These cells injected into the hamster formed a ductal-type adenocarcinoma. Compared to the well-differentiated TAKA-1-BOP tumor, which was induced by injection of BOP treated hamster ductal cells *in vitro* [15], the KL5B tumor was anaplastic and highly invasive. Contrary to TAKA-1-BOP cells, KL5B cells showed K-*ras* mutation and more extensive chromosomal alterations. In particular, the missing Y chromosome is noteworthy, because ILA tumor cells [28], which were induced by BOP in a hamster submandibular gland bearing a homologous islet transplant, also showed this abnormality. Also noteworthy is that the missing Y chromosome has been found to be one of the most consistent abnormalities of human pancreatic cancer [29]. Whether this abnormality is associated with the malignancy of specific pancreatic cells remains to be seen.

The invasive nature of the KL5B- and ILA tumor cells could be related to the exposure of evolving tumor cells within islets to a high concentration of growth factors. Moreover, simultaneous expression of vimentin and cytokeratines in KL5B cells, also found in aggressive human breast cancer [30] seems to be associated with invasive behavior. The same evidence seems to apply to the lack of blood group A antigen expression. We have shown that well-differentiated, BOP-induced tumors and the slow growing PC-1 cell line derived from a primary BOP-induced cancer [14], consistently express blood group A antigen [31]; whereas, the poorly differentiated, fast-growing PC-1.0

Table 1: Immunohistochemical reactivity of antibodies to normal hamster pancreas, KL5N and KL5B cells (day 224)

Antibody	Normal pancreas	KL5N	KL5B
Pancytokeratin	+++[a,b]	+++[a] (30)	+++[a] (20)
Cytokeratin 14	+++[*,a]	++[a] (20)	++[a] (10)
Cytokeratin 18	++[*,a]	+++[a] (5)	+++[a] (10)
Carbonic anhydrase II	+[*]	+ (20)	+[a] (20)
Laminin	++[a,b] (100)	+++[a] (50)	+++[a] (100)
Vimentin	+++[c] (100)	+++[a] (70), +(20)	+++[a] (100)
Tomato lectin	+++[e] (100)	+++[a,d] (100)	+++[d,f] (100)
PHA-L	+++[f] (100)	+++[d] (100)	+++[d] (100)
TGF-α	+[g] (20)	++[a] (30)	+ ++[a] (70)
EGFR	-	+[d] (10)	+ +[a] (80)
α-1-Antitrypsin	+[b] (100)	+[a,d] (90)	+++[a,d] (100)

(%) percentage of stained cells; -, No staining; +, weak staining; ++, moderate staining; +++, strong staining
[a], diffuse cytoplasmic; [b], acinar, islet-and ductal cells staining; *, staining of ductal cells only; [c], staining of smooth muscles only;
[d], cell membrane staining; [e], diffuse cytoplasmic staining of zymogen granules and of islet cells, luminal staining of ductal cells;
[f], diffuse cytoplasmic staining of zymogen granules and islet cells; [g], staining of glucagon cells only.

Table 2: Immunohistochemical reactivity of antibodies to KL5B cells (day 224) injected subcutaneously, and into the submandibular gland (SMG) or pancreas

Antibody	Subcutaneously	SMG	Pancreas
Pancytokeratin	+^{a} (10)	+ to ++^{a} (10)	+ to ++^{a} (20)
Cytokeratin 14	+^{a} (5)	+^{a} (5)	+^{a} (2)
Cytokeratin 18	+^{a} (10)	+^{a} (10)	+^{a} (10)
Carbonic anhydrase	-	+^{a} (4)	+^{a} (5)
Laminin	+ ^{a} (100)	+^{a} (100)	+ to +++ $^{a, b}$ (100)
Vimentin	+ to +++ ^{c} (100)	+++^{c} (100)	+ to ++ ^{c} (80)
Tomato lectin	++ to +++ ^{d} (100)	+ to +++^{b} (100)	+ to ++ ^{d} (100)
PHA-L	+ ^{e} (100)	+++^{c} (100)	+++ ^{c} (100)
EGFR	+ ^{a} (30)	+^{a} (60)	+ +^{a} (80)
TGF-α	+ ^{a} (40)	+^{a} (65)	+ +^{a} (80)
α-1-Antitrypsin	+ ^{a} (70)	++^{a} (80)	+ ^{a} (60)

(%) percentage of cells stained; -, No staining; +, weak staining; ++, moderate staining; +++, strong staining. ^{a}, diffuse cytoplasmic; ^{b}, weak staining of tumor cells, strong staining of blood vessels; ^{c}, granular cytoplasmic staining; ^{d}, cell membrane and vascular staining; ^{e}, cell membrane staining

cells, derived from a subcutaneous transplant of a primary hamster pancreatic cancer, do not. This finding may indicate that the blood group antigen represents a differentiation marker. However, contrary to PC-1.0 cells, which assume blood group A antigen expression when transplanted into hamsters [16], KL5B cells failed to produce this antigen *in vivo*. This may be related to the extensive genetic changes in KL5B cells. On the other hand, the expression of α-1-antitrypsin, in both KL5N and KL5B cells but not in any adult hamster pancreatic cells, may support the origin of these cells from a primitive precursor cell. This acute-phase reactant protein has been found in human pancreatic tumors assumed to derive from stem cells, including solid cystic (papillary) tumor [32] and pancreatoblastoma [33]. Hence, this protein, as well as vimentin, not present in pancreatic parenchymal cells, appears also to represent a marker for hamster pancreatic stem cells [34]. Although it appears that tumors originating from ductal cells or from within islets, have different biological appearance, confirmatory studies are required.

The malignant alteration of islets by BOP in culture unequivocally points to the ability of islet cells to metabolize BOP. Consequently, it appears that both, hamster ductal cells [15], and islets can be transformed by BOP and both have the necessary enzymes to metabolize BOP.

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