Short Communication

In Vitro Induction of Giant Cell Tumors from Cultured Hamster Islets Treated with *N*-Nitrosobis(2-Oxopropyl)amine

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Giant cell carcinoma of the pancreas is a rare tumor. Its histogenesis is still controversial. In a Syrian hamster pancreatic cancer model, tumors similar to human giant cell carcinomas have been induced at an extremely low rate of incidence and after the use of high doses of pancreatic carcinogens. Thus far no tumors of giant cell type have been induced by the *in vitro* treatment of hamster pancreatic ductal cells with the potent pancreatic carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP). In the present study we report the induction of giant cell carcinoma from hamster islets treated with BOP *in vitro*. The results suggest that in hamsters some component of islet cells, probably stem cells, are the origin of giant cell carcinoma. (Am J Pathol 2000, 156:439–443)

It is generally believed that over 90% of pancreatic cancers originate from ductal/ductular cells. Studies of the hamster pancreatic cancer model, which in biological and morphological aspects mimics human tumors,^{1–3} however, have shown that pancreatic ductal adenocarcinoma originates not only from ductal/ductular cells but primarily from within islets, most probably from precursor (stem) cells.^{1–3} In this model, tumors resembling human giant cell carcinoma have been induced only after prolonged treatment with high doses of pancreatic carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP).⁴ In humans, giant cell carcinoma is also a rare tumor and appears as

two different phenotypes, osteoclastic and pleomorphic. The histogenesis of these tumors from epithelial or mesenchymal cells has not yet been settled. Treatment of isolated hamster pancreatic ductal cells with BOP or methylnitrosourea (MNU) *in vitro* resulted in the induction of anaplastic cancer, but no giant cell tumors have been observed.^{5,6} A similar treatment scheme of isolated purified hamster pancreatic islets with BOP, however, resulted in formation of anaplastic invasive cancers, which, *in vitro* and *in vivo*, presented patterns consistent with human pancreatic giant cell tumor. In this report we describe some biological patterns of this tumor compared to the human tumor.

Materials and Methods

Animals

Female and male Syrian golden hamsters (all 8–10 weeks old) from the Eppley colony and 8-week-old female BEIGE DX 1 nude mice from Harlan Comp. (Indianapolis, IN) were used in this experiment. The animals were housed in cages with Sani-cell bedding and kept under standard laboratory conditions (temperature 20 ± 2°C, 10 air changes/minute, 12 hour/12 hour light/dark cycle). They received a pelleted diet (Wayne, Indianapolis, IN) and water *ad libitum*.

Chemicals

BOP was synthesized at our institute. The culture medium (M3:5) was a gift from InCell Corporation (San Antonio,

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Table 1. Immunohistochemical Reactivity of Antibodies to Hamster-Islet-Derived Cell Line MS7N and MS7B at 40 Weeks *in Vitro* and *in Vivo*

Antibody	Dilution	Hamster pancreas (control)	MS7N	MS7B* (<i>in vitro</i>)	MS7B tumor* (<i>in vivo</i>)
Pancytokeratin	Ready to use [†]	++ ^{§¶}	+++ [§]	++ [§]	++ [§]
NSE	Ready to use	++ [§]	++ [§]	++ [§]	++ [§]
TGF- α	1:20***	+ [§]	+ [§]	+ [§]	+ [§]
EGFR	1:40***	—	—	+**	+**
Vimentin	Ready to use	++ ^{††}	+ [§]	++ [§]	++ [§]
Laminin	1:25	++ ^{§¶}	++ [§]	++ [§]	++ [§]
Tomato lectin	1:200	+++ ^{††}	+++ ^{§**}	+++ ^{§¶}	+++ ^{§**}
L-PHA	1:200	+++ ^{§§}	+++ ^{**}	+++ ^{**}	+++ ^{**}
α -1 Antitrypsin	Ready to use	+ ^{§**}	+ ^{§**}	+ ^{§**}	+ ^{§**}
p53 (Pab 240)	1:50	—	—	++ [§]	++ [§]

—, no staining; +, weak staining; ++, moderate staining; +++, strong staining.

*Including giant cells.

[†]Pretreatment (0.2% pepsin in 0.01 N HCl) was performed for 30 minutes at 37°C.

[‡]Pretreatment (0.05% of saponin (w/v) diluted with Tris-buffered saline) was performed for 30 minutes at room temperature.

[§]Diffuse cytoplasmic staining.

[¶]Staining of ductal, ductular, centroacinar, and islet cells.

^{||}Staining of islet cells only.

^{**}Cell membrane staining.

^{††}Staining of smooth muscle only.

^{‡‡}Diffuse cytoplasmic staining of zymogen granules and islet cells, luminal staining of ductal cells.

^{§§}Diffuse cytoplasmic staining of zymogen granules and islet cells.

TX). Penicillin, streptomycin, trypsin, EDTA, and RPMI-1640 were purchased from Life Technologies (Gaithersburg, MD). Fetal bovine serum was purchased from Summit Biotechnology (Ft. Collins, CO). Collagenase P was obtained from Boehringer Mannheim (Mannheim, Germany).

Islet Cell Culture and BOP Treatment

Pancreatic islets of 10 male Syrian golden hamsters were isolated, purified, and cultured as described earlier.⁷ Briefly, surgically removed pancreata were digested with 5 mg/ml collagenase P for 10 minutes, and islets were hand-picked under a dissecting light microscope. For the first 14 days the islets were maintained on a rocker (20 rpm) in 60-mm Petri dishes containing 8 ml M3:5 medium in a humid atmosphere and 5% CO₂. While the islets were floating, the fibroblasts attached to the bottom of the dishes. Islets were hand-picked every other day and placed in a new Petri dish with fresh M3:5 medium. At day 15, after the initial isolation, pure islets were plated in T-25 flasks (about 100 islets per flask) and allowed to attach to the bottom of the flask. From attaching islets, epithelial cells radiated into the surrounding area and formed a monolayer. The cells were trypsinized and subcultured when subconfluent. Half of the islets were treated with BOP from the first day of culture at a concentration of 0.25 mmol/L every other day, three times a week, and were designated as MS7B. The other half served as an untreated control group (MS7N). After 27 weeks in culture, the cells were gradually shifted from M3:5 medium to 1640-RPMI containing 10% fetal bovine serum. Cell growth speed and anchorage-independent cell growth were determined as reported.⁵

In Vivo Cell Growth

A total of 6×10^6 MS7B and MS7N cells from the same passages were injected subcutaneously into two sites of

the abdomen of four Syrian golden hamsters and four nude mice. The tumor growth was measured weekly and bidimensionally with a caliper. Tumor-bearing animals were euthanized after 4 weeks. In animals with MS7N, transplants were observed for 10 weeks. The tumors were freed from soft tissues, weighed, measured, fixed in 10% buffered formalin for 16 hours, and processed for histology by conventional methods.

Histology/Immunocytochemistry

At weeks 27 and 40, 5.0×10^6 MS7B and MS7N cells were washed in $1 \times$ phosphate-buffered saline, fixed in 10% buffered formalin for 1 hour, embedded in paraffin, and cut into 4- μ m serial sections. Cells of both groups were also cultured in four-well chamber slides (Nalge Nunc International, Naperville, IL) and in 12-well plates on Thermanox plastic coverslips (Nalge Nunc International) and fixed with 10% buffered formalin for 1 hour. The cells were stained with hematoxylin and eosin (H&E). For immunocytochemistry, the avidin-biotin peroxidase complex (ABC) method was used, and the antibodies were visualized with diaminobenzidine (DAB) peroxide (KPL, Gaithersburg, MA). The primary antibodies used in this study were as follows: insulin, glucagon, somatostatin, and p53 (Pab240) purchased from Zymed (San Francisco, CA), neuron-specific enolase (NSE), vimentin and α -1-antitrypsin from Biogenex (San Ramon, CA), pancytokeratin and laminin from Sigma (St. Louis, MO), and transforming growth factor- α (TGF- α) and epidermal growth factor receptor (EGFR) from Oncogene Science (Cambridge, MA). Tomato lectin and *Phaseolus vulgaris* leucoagglutinin (L-PHA) were purchased from Vector (Burlingame, CA). The primary antibodies were diluted as shown in Table 1. A normal hamster pancreas served as a control. Negative control sections were processed similarly, except that nonimmunized serum of the host animal or type-specific immunoglobulin was used instead of the

primary antibody. The staining intensity was scored as negative (-), weak (+), moderate (++), and strong (+++).

Electron Microscopy

At weeks 27 and 40, 1.0×10^6 MS7B and MS7N cells were each prepared for transmission electron microscopic examination as reported.⁸

Examination of Ki-ras Mutation

Mutation of the Ki-ras gene in MS7B and MS7N cells at weeks 27 and 40 by reverse transcriptase-polymerase chain reaction, and the sequence analysis was performed as reported.^{5,9} The primer sequences used are listed elsewhere.⁹

Results

The patterns of BOP-treated and untreated islets in culture and their subsequent cytological changes were similar to those published earlier.^{7,10} The sizes of the treated and untreated islets varied between 0.12 and 0.32 mm without significant difference between the two groups. After the islets were transferred into the flasks, all islets attached to the bottom of the flasks within a week and a large number of epithelial cells radiated from the islet cores into the surrounding area as reported.^{7,10} These epithelial cells were gradually replaced by monomorphic undifferentiated cells. At 11 weeks and later, contrary to the monomorphic appearance of the MS7N cells, the MS7B cells became pleomorphic, with abundant eosinophilic cytoplasm and a few hyperchromatic nuclei surrounded by fine granules.

After 36 weeks in culture, mononucleated or polynucleated giant cells appeared for the first time. The polynucleated cells had three to eight overlapping nuclei in a large polygonally shaped cytoplasm that was 10–20 times larger in size than the surrounding undifferentiated cells (Figure 1). MS7N cells retained their monomorphic phenotype throughout the whole culture.

After 13 weeks, the growth of MS7B cells accelerated significantly compared to the MS7N cells, showing a doubling time of 30 hours compared to 36 hours for the MS7N cells. The doubling time of untreated and treated cells further decreased with time in culture. After 40 weeks in culture the cell growth of both groups remained fairly constant, with a doubling time of 11 hours for the MS7B cells and 21 hours for the MS7N cells.

At 27 weeks in culture BOP-treated cells but not MS7N cells formed for the first time 12 colonies per dish in soft agar. In *in vivo* experiments, these MS7B cells also formed subcutaneous tumors for the first time. At this stage, tumors grew to an average size of 0.32 cm³ within 3 weeks in both species. Cells of later stages grew more aggressively and formed tumors of 1.72 cm³ within 3 weeks on average. MS7N cells at the same stages as the MS7B cells did not form tumors within a period of 10

Figure 1. MS7B cells at 40 weeks in culture, grown on a chamber slide. **a:** Scattered between small cells in haphazard order, there are scattered mononucleated or polynucleated giant cells with large cytoplasm. H&E; original magnification, $\times 75$. **b:** Higher power view of polynucleated giant cells, showing multiple nuclei that were either separated from each other (**top**) or presented more than 10 overlapping nuclei (**bottom**). H&E; original magnification, $\times 140$.

weeks. There were no differences between the tumors grown in hamsters and nude mice. Histologically, both hamster and mouse tumors were composed primarily of pleomorphic spindle-shaped cells, forming an anaplastic tumor but no giant cell tumor. Tumors from MS7B cells treated with BOP for 40 weeks were primarily composed of anaplastic cells with scattered pleomorphic multinucleated giant cells (Figure 2). In nude mouse and hamster, eight tumors were found, and all eight were giant cell tumors.

Immunohistochemical results of MS7N and MS7B cells at 40 weeks in culture *in vitro* are shown in Table 1. MS7N and MS7B cells, the MS7B cells growing as tumors *in vivo*, as well as the giant cells *in vitro* and *in vivo*, stained positively with anti-pancytokeratin, -NSE, -TGF- α , -vimentin, -laminin, and bound to tomato lectin and L-PHA. Reactivity to EGFR staining was found only in MS7B cells after 40 weeks in culture. p53 overexpression was found

Figure 2. MS7B cells at 40 weeks in culture, grown in a nude mouse. The tumor is composed of pleomorphic small cells with interspersed mononucleated or polynucleated cells, some with large and almost homogeneous cytoplasm. H&E; original magnification, $\times 210$.

Figure 3. Electron microscopically, the giant cells had several elongated nuclei with irregular contours, small hyperchromatic nucleoli, and sparse organelles and lysosomes. At week 40 in culture. Original magnification, $\times 4500$.

only in MS7B at week 40. None of the cells reacted to anti-insulin, -glucagon, or -somatostatin.

Electron microscopically, the cells treated with BOP for 24 weeks had a pleomorphic shape and were 5–200 μm in diameter. They were loosely arranged and occasionally attached to each other. They also presented oval or round mitochondria, chromatin-rich irregular nuclei, and large nucleoli. Some cells had intracytoplasmic cystic spaces filled with debris. After 40 weeks in culture, scattered polynucleated giant cells 600–800 μm in diameter were observed. They contained three to five large nuclei, many lysosomes, and sparse rough endoplasmic reticulum (Figure 3).

Mutation of the *K-ras* oncogene was found in the MS7B giant cells in culture but not in the MS7N cells. The mutation was in codon 12 (GGT $\rightarrow$ GAT).

Discussion

Giant cell tumor of the pancreas is a rare tumor with an incidence of 2.1–12.8%^{11,12} and has a grave prognosis.¹³ Histologically, two types of giant cell tumors are distinguished: pleomorphic and osteoclastic. Pleomorphic giant cell tumor, also termed *carcinosarcoma*, is considered to be a sarcomatous metaplasia of ductal adenocarcinoma^{14–16}; the origin of osteoclastic giant cell tumor from epithelial or mesenchymal cells is still debated.¹⁴ Their differential diagnoses include malignant fibrous histiocytoma, amelanotic melanoma, soft tissue sarcoma, and hepatocellular carcinoma. Because of their morphological and biological patterns these tumors were initially regarded as a separate type of pancreatic cancer. Although the new classification system includes

these tumors in the anaplastic category, their etiology and histogenesis have remained obscure.

In the hamster pancreatic cancer model, which in many aspects resembles the human disease,^{1,2,9,17} both pleomorphic giant cell tumor and osteoclastic giant cell tumor have been induced.⁴ However, the cell origin of these neoplasms could not be ascertained. Although in humans most cancer types, including pleomorphic giant cell tumors, are thought to derive from ductal cells, in the hamster model many cancers originate from within islets,^{1–3} most probably from stem cells.¹⁰ In contrast to tumors arising from ducts (intraductal carcinomas), which remain within the ductal boundary for a long time before invasion, tumors developing within islets (intra-islet cancers) are invasive from their inception.¹⁰ We and others have shown that *in vitro* treatment of hamster ductal cells with pancreatic carcinogens leads to malignant cell transformation and adenocarcinoma *in vivo*.^{5,6} No giant cell carcinomas have ever been produced. On the contrary, treatment of isolated and purified hamster islets with BOP produced tumors of giant cell morphology. Similarities between the human and hamster tumors exist also on immunohistochemical and biological levels, including the coexpression of the epithelial marker cytokeratin, mesenchymal marker, vimentin,^{14,18,19} neuroepithelial marker, NSE,¹³ the mutation of *K-ras*^{18,19} and overexpression of *p53*.²⁰ P16 and DPC4 mutations reported in human pancreatic cancer^{21,22} could not be examined because of the unavailability of the respective primers for hamsters (see comments in Ref. 22).

Opinions on the progenitor cells of giant cell tumor are divided. Although most investigators believe that the giant cells derive from ductal cells,²³ others assume that pancreatic precursor cells are the origin of the tumor.²³ The latter possibility is supported by the occurrence of mixed ductal adenocarcinoma-pleomorphic giant cell tumor on the one hand and osteoclastic and pleomorphic giant cells on the other.^{15,24} Our finding supports the precursor (stem) cell derivation of giant cell carcinoma. Because the tumor was induced only when islet and not ductal cells were treated with the carcinogen, we concluded that some cells within islets present the tumor progenitor cells. We have identified these cells in culture and have shown their response to BOP.^{7,10} The reactivity of giant cells with NSE (a marker for neuroendocrine differentiation) and $\alpha 1$ -antitrypsin, which has been shown to be expressed in human cancer cells assumed to derive from pancreatic stem cells, such as pancreatoblastoma and solid cystic tumors,^{25–27} also points to their stem cell derivation.

Nevertheless, we have shown for the first time the induction of pleomorphic giant cell cancer from cultured hamster islets treated with a potent pancreatic carcinogen that they derive from stem cells embedded within islets.

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