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ORIGINAL ARTICLE

Common Bile Duct Injection as a Novel Method for Establishing Red Fluorescent Protein (RFP)-Expressing Human Pancreatic Cancer in Nude Mice

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ABSTRACT

Context In our previous pancreatic cancer mouse models, we have used surgical orthotopic implantation of human pancreatic tumors to establish clinically relevant fluorescent mouse models of pancreatic cancer.

Objective Since exocrine pancreatic cancer is thought to arise from the cells lining the ducts of the pancreas, we hypothesized that direct injection of tumor cells into the common bile duct would also result in pancreatic tumor formation and metastasis.

Intervention In this study we injected a suspension of the low passage human pancreatic cancer cell line xPA-1 transfected with red fluorescent protein into the common bile duct of nude mice.

Main outcome measure Pancreatic tumor growth and metastasis formation was monitored by intravital and whole body fluorescent imaging. Single fluorescent pancreatic cancer cells were imaged in the pancreatic duct shortly after injection using the Olympus OV100 Whole Mouse Imaging System.

Results Five days after tumor cell injection in the common bile duct, tumor colonies could be imaged forming within the pancreatic duct. Metastases in the liver were imaged 14 days post common bile duct injection. By day 28, massive tumors were imaged encompassing the entire pancreas. By day 42, RFP-expressing metastases were imaged in the omentum and liver.

Conclusion Common bile duct injection is a novel technique for the development of fluorescent mouse models of metastatic pancreatic cancer.

INTRODUCTION

We have previously described the development and use of orthotopic nude mouse models of pancreatic cancer [1, 2, 3]. To visualize the metastatic pattern of pancreatic cancer *in vivo*, we developed stable high-expression green fluorescent protein (GFP) transductants of human pancreatic cancer cell lines [2, 3]. Fragments of subcutaneous-growing tumors were implanted by surgical orthotopic implantation (SOI) onto the pancreas of nude mice. Subsequent micrometastases were visualized by GFP

fluorescence in the peritoneum, periportal lymph nodes, liver, and lung, as well as other sites in the abdominal cavity. The use of GFP-expressing MiaPaCa-2 and BxPC-3 cells transplanted by SOI revealed the extensive metastatic potential of pancreatic cancer at the cellular level *in vivo* [2, 3]. Furthermore, the primary tumor and subsequent metastasis were visualized by whole body imaging through the skin of the nude mouse without the need for laparotomy [2]. Such visualization was a practical and convenient way to follow metastasis in a "real-time" fashion. Subsequently, this metastatic model played a critical role in the study of the mechanism of metastasis in pancreatic cancer and in screening of therapeutics that prevent or reverse this process [4, 5].

As an alternative to the GFP model, we subsequently described an orthotopic highly metastatic model of pancreatic cancer that utilizes pancreatic cancer cells engineered to express very high levels of the *Discosoma sp.* red fluorescent protein (RFP) [4, 6, 7]. As in the GFP models, this model also resembles human pancreatic cancer in its pattern of growth and metastasis. It rapidly and reliably produces distant metastatic disease, and frequently gives rise to malignant abdominal ascites and peritoneal carcinomatosis. Moreover, the enhanced fluorescence of this model enables sensitive, real-time whole body imaging of pancreatic tumor growth and metastasis in the live animal. This model permits visualization of both macro- and micrometastases. These features make the model an ideal system with which to study the effects of novel antineoplastic agents on tumor growth and metastasis [4, 6, 7].

Recent imaging technology has enabled *in vivo* imaging at the single-cell level [8]. For single-cell imaging on deep organs, reversible skin-flaps and chronic windows can be used. Single-cell imaging can be used to study cancer cell invasion, seeding in distant organs and dormancy. In the present study, we used single cell imaging technology with the Olympus whole mouse imager to visualize tumor events following direct injection of fluorescent human pancreatic cancer cells into the common bile duct of nude mice. These routes of injection were chosen because they are clinically important routes of metastasis in pancreatic cancer.

METHODS

Cell Culture

Low-passage human pancreatic cancer cells, XPA-1, were established at the John Hopkins University Baltimore, MD, USA. The cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine (Gibco-BRL, Life Technologies, Inc., Grand Island, NY, USA). The cell line was cultured at 37°C in a 5% incubator. The XPA-1 line was used, because it is a low passaged human pancreatic cancer patient cell line.

Red Fluorescent Protein Vector Production [9]

The RFP (DsRed-2) gene (BD Biosciences Clontech, Palo Alto, CA, USA) was inserted in the retroviral-based mammalian expression vector pLNCX (BD Biosciences Clontech, Palo Alto, CA, USA) to form the pLNCX DsRed-2 vector. Production of retrovirus resulted from transfection of pLNCX DsRed-2 in PT67 packaging cells, which produced retroviral supernatants containing the DSRed-2 gene. Briefly, PT67 cells were grown as monolayers in DMEM supplemented with 10% FCS (Gemini Biological Products, Calabasas, CA, USA). Exponentially growing cells (in 10 cm dishes) were transfected with 10 µg expression vector using a Lipofectamine Plus (GIBCO-BRL, Grand Island, NY, USA) protocol. Transfected cells were replated 48 hours after transfection and 100 µg/mL G418 was added 7 hours after transfection. Two days later, the amount of G418 was increased to 200 µg/mL G418. After 25 days of drug selection, surviving colonies were visualized under fluorescence microscopy and RFP-positive colonies were isolated. Several clones were selected and expanded into cell lines after virus titering on the 3T3 cell line.

RFP Gene Transduction of Tumor Cell Line [9]

For RFP gene transduction, 20% confluent XPA-1 human pancreatic cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI 1640 or other culture media (GIBCO-BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (Gemini Biological Products, Calabasas, CA, USA) for 72 hours. Fresh medium was replenished at this time. Tumor cells were harvested with trypsin/EDTA and subcultured at a ratio of 1:15 into selective medium, which contained 50 µg/mL G418. To select brightly fluorescent cells, the level of G418 was increased to 800 µg/mL in a stepwise manner. RFP (DsRed-2) is not toxic to cells at any concentration of G418. Clones expressing RFP were isolated with cloning cylinders (Bel-Art Products, Inc., Pequannock, NJ, USA) by trypsin/EDTA and were amplified and transferred by conventional culture methods in the absence of selective agent.

Animals

Thirty athymic *nu/nu* nude mice between 4 and 6 weeks of age were maintained in a barrier facility on high efficiency particulate air (HEPA)-filtered racks. The animals were fed with autoclaved laboratory rodent diet (Teckland LM-485; Western Research Products, Orange, CA, USA). Fluorescent human pancreatic cancer cells were directly injected into the common bile duct of nude mice.

Fluorescent Optical Imaging and Processing [8]

The Olympus OV100 Whole Mouse Imaging System (Olympus Corp., Tokyo, Japan), containing an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD camera (Olympus Corp., Tokyo, Japan) was used for subcellular imaging in live mice. The optics of the OV100 fluorescence imaging system have been specially developed for macro as well as micro imaging with high light gathering capacity, and incorporate a unique combination of high numerical aperture and long working distance. Five individually optimized objective lenses, parcentered and parfocal, provide a 105-fold magnification range for seamless imaging of the entire body down to the sub-cellular level without disturbing the animal. The OV100 has the lenses mounted on an automated turret with a magnification range of 1.6X to 16X and a field of view ranging from 6.9 mm to 0.69 mm. The optics and anti-reflective coatings ensure optimal imaging of multiplexed fluorescent reporters in small animals. High-resolution images were captured directly on a PC (Fujitsu Siemens, Munich, Germany). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and Cell® (Olympus Biosystems, Planegg, Germany) [8].

Injection of Cancer Cells into the Common Bile Duct

Nude mice were anesthetized with ketamine by injection into the muscle of a lower limb.

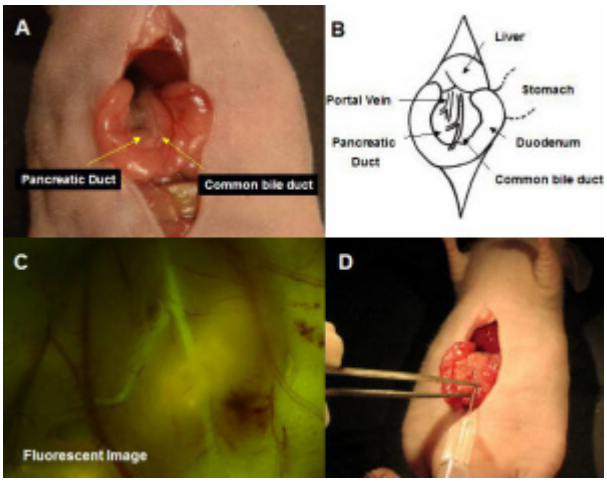

Figure 1. Technical aspects of common bile duct injection. **a.** Gross anatomy of the common bile duct and pancreatic duct in the nude mouse as seen through an upper midline laparotomy incision. **b.** Schematic depiction of anatomical structures seen in panel a. **c.** The common bile and pancreatic ducts can be imaged with autofluorescence if the anatomy is unclear. **d.** Injection of the common bile duct with pancreatic tumor cells using a 27 gauge needle.

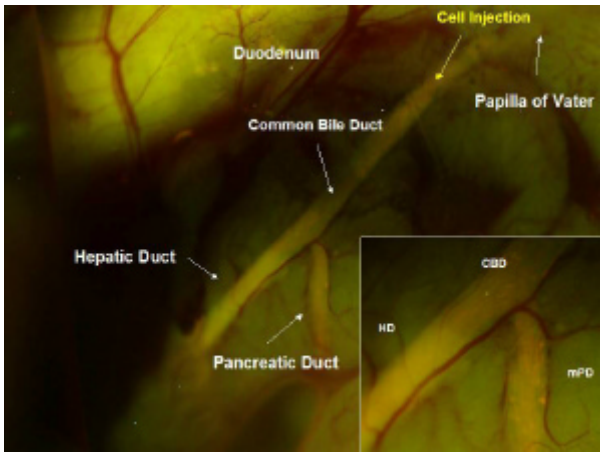

Figure 2. Single cell imaging immediately after injection of XPA-1 red fluorescent protein (RFP) human pancreatic cancer cells into the common bile duct. RFP human pancreatic tumor cells can be imaged within the common bile ducts and pancreatic ducts using the OV100 imaging system. Inset, magnified view of the major pancreatic duct (mPD), common bile duct (CBD), and hepatic duct (HD).

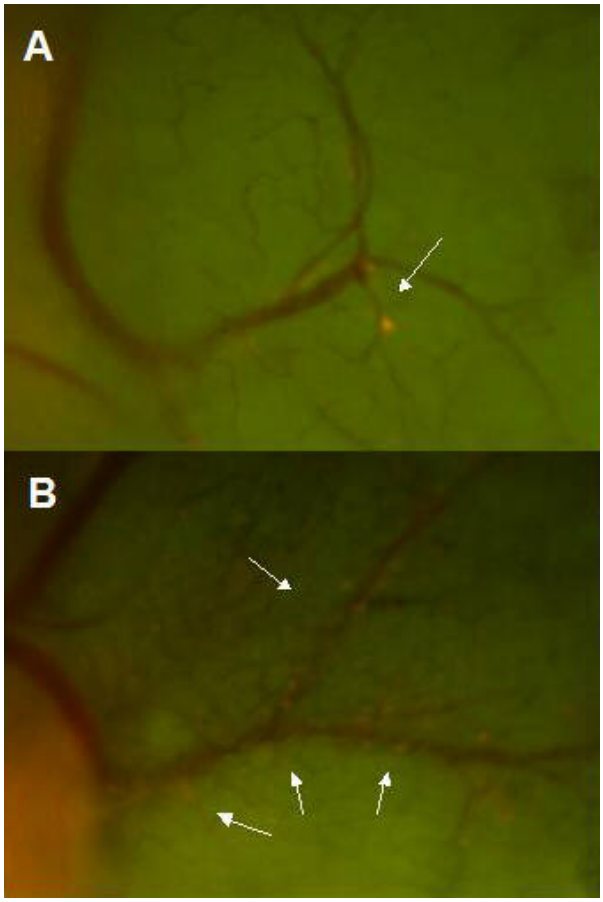

Figure 3. a. XPA-1 red fluorescent protein (RFP) tumor cells imaged at the single cell level (arrow). **b.** XPA-1 RFP cells imaged within the peripheral pancreatic ducts (multiple arrows).

An incision was created through the middle upper abdominal line and the duodenum and pancreatic head were carefully exposed. The common bile duct was identified and injected with xPA1-RFP cells ($1.0x10^6$/50 μL) using a 27G needle (Figure 1). The injection procedure was carried out slowly and gently with no apparent injury. The abdominal wall and the skin were closed in 1 layer using 6-0 surgical sutures. All procedures were performed with a 7X microscope (Olympus America, Inc., Melville, NY, USA) or standard surgical loupes. The tumor take rate was 100%.

ETHICS

All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals (1996)" prepared by the National Academy of Sciences.

RESULTS

Single Cell Imaging of Fluorescent Human Pancreatic Tumor Cells within the Common Bile and Pancreatic Ducts

Single cells can be seen within the lumen of the pancreatic duct immediately after injection of XPA-1 RFP human pancreatic cancer cells into the common bile duct (Figure 2). RFP human pancreatic tumor cells were imaged within the common bile ducts and pancreatic ducts using the OV100 Whole Mouse Imaging System. The cells quickly traveled and disbursed to the peripheral pancreatic ducts immediately after injection where they eventually formed tumor colonies (Figure 3). No extra-pancreatic cancer cells nor ascites were detected right after injection, indicating the orthotopic injections were successful.

Pancreatic Tumor Colony Formation

Tumor colonies began to form by post injection day 5 (Figure 4a,b). By 2 weeks, large tumors were seen growing within the

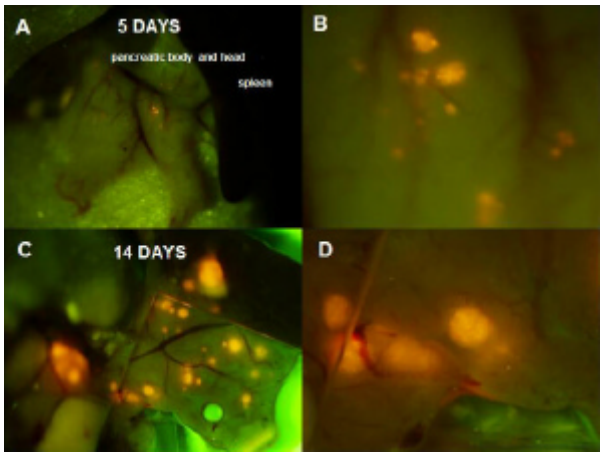

Figure 4. Tumor colonies are beginning to form by post injection day 5 (panel **a; b**: magnified view) and day 14 (panel **c; d:** magnified view).

pancreatic parenchyma (Figure 4c,d). The use of RFP allowed visualization of the extent of tumor and metastatic growth. The cell kinetics observed are cell-line specific and not due to RFP or GFP.

Late Stages of Primary Pancreatic Cancer and Metastases

By day 28 post injection, a large red fluorescent pancreatic tumor was seen (Figure 5a) and a fluorescent liver metastasis was also imaged (Figure 5b). By day 42, the entire pancreas and omentum is replaced by tumor (Figure 5c). The survival rate is approximately 50 days. An image of an opened mouse shows omental tumor and multiple liver and lung metastases (Figure 5d).

DISCUSSION

In this report, we describe a novel method of establishing pancreatic cancer in nude mice using common bile duct injection of fluorescent pancreatic cancer cells. Since the most common type of exocrine pancreatic cancer is ductal adenocarcinoma and cancer begins in the cells that line the pancreatic duct, this route of delivery of tumor cells adheres to the seed-and-soil hypothesis of Paget [10]. Ductal adenocarcinomas of the pancreas grow rapidly and regardless of tumor site, most patients have evidence of spread at the time of diagnosis and at operation [11, 12]. Invasion of contiguous organs by direct extension is a characteristic feature of pancreatic ductal adenocarcinoma and was also seen in our model [13]. The intraductal model represents advanced metastatic cancer in the patients where multiple metastatic events may occur. Lymphatic spread to the different peripancreatic lymph node groups is common and hepatic blood borne metastases are frequent. These features of human pancreatic cancer were imaged in our model late in the course of the disease.

With the possibility of *in vivo* subcellular dual-color imaging of fluorescent tumor cells in the live animal, the biology and behavior of cancer cells can more accurately be studied [8]. The high extinction coefficients, quantum yields, and unique spectral properties of fluorescent proteins have been taken advantage of in order to visualize, in real time, the important aspects of cancer in living animals, including tumor cell trafficking, invasion, metastasis, and angiogenesis [14]. Fluorescent proteins enable whole-body imaging of tumors on internal organs. These multicolored proteins have allowed the color-coding of cancer cells growing *in vivo* with distinction of different cell types, including host from tumor, with single-cell resolution [15, 16, 17]. The fluorescent model of pancreatic cancer described in these experiments should be very useful in defining

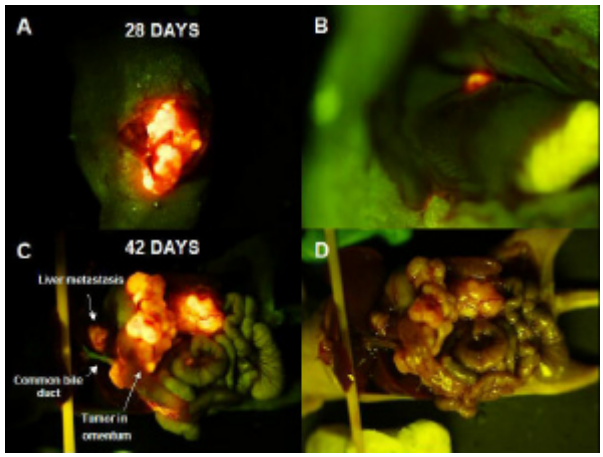

Figure 5. a. By day 28 post injection, a large red fluorescent pancreatic tumor is seen. **b.** A fluorescent liver metastasis is also imaged (day 28). **c.** By day 42, the entire pancreas and omentum is replaced by tumor. **d.** Gross view of same mouse in panel C (day 42).

cellular trafficking and targeting mechanisms of pancreatic cancer metastasis.

CONCLUSIONS

A low passaged cell line established from a pancreatic cancer patient specimen was transfected with RFP and injected into the common bile duct of nude mice to establish a ductal model of pancreatic cancer. Imaging was enabled with the Olympus OV100 whole-mouse imaging system with both macro and objective lens micro optics. The injected cancer cells were immediately imaged to translocate to the peripheral pancreatic ducts. The tumor cells established colonies in the head, body, and tail of the pancreas and metastasized to the omentum and liver. This new imageable model of pancreatic cancer should be useful for visualizing specific steps of metastases in real time and to selectively target drugs to these steps.

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Keywords Common Bile Duct; Mice, Nude; Pancreatic Neoplasms; red fluorescent protein

Abbreviations GFP: green fluorescent protein; HEPA: high efficiency particulate air; RFP: red fluorescent protein; SOI: surgical orthotopic implantation

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