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In vivo internal tumor illumination by telomerase-dependent adenoviral GFP for precise surgical navigation

Hiroyuki Kishimoto, Ming Zhao, Katsuhiro Hayashi, Yasuo Urata, Noriaki Tanaka, Toshiyoshi Fujiwara, Sheldon Penman, and Robert M. Hoffman

Cancer surgery requires the complete and precise identification of malignant tissue margins including the smallest disseminated lesions. Internal green fluorescent protein (GFP) fluorescence can intensely illuminate even single cells but requires GFP sequence transcription within the cell. Introducing and selectively activating the GFP gene in malignant tissue in vivo is made possible by the development of OBP-401, a telomerase-dependent, replication-competent adenovirus expressing GFP. This potentially powerful adjunct to surgical navigation was demonstrated in 2 nude mouse models that represent difficult surgical challenges—the resection of widely disseminated cancer. HCT-116, a model of intraperitoneal disseminated human colon cancer, was labeled by virus injection into the peritoneal cavity. A549, a model of pleural dissemination of human lung cancer, was labeled by virus administered into the pleural cavity. Only the malignant tissue fluoresced brightly in both models. In the intraperitoneal model of disseminated cancer, fluorescence-guided surgery enabled resection of all tumor nodules labeled with GFP by OBP-401. The data in this report suggest that adenoviral-GFP labeling tumors in patients can enable fluorescence-guided surgical navigation.

Adenovirus | green fluorescent protein | metastasis

The intent of cancer surgery is to remove malignant tissue together with margins of presumably normal tissue (1–3) to ensure complete removal of abnormal cells. Estimating margin width during surgery is critical and depends on the surgeon’s vision. There have been many developments intended to improve the delineation of tissue margins using morphologic and optical differences between normal and abnormal tissue. This report describes a major enhancement of cancer surgical navigation using the selective fluorescent labeling in vivo, of malignant tissue. Bright GFP fluorescence clearly illuminates the tumor boundaries and facilitates detection of the smallest disseminated disease lesions.

Highly selective viral replication in malignant cells growing in normal tissue has recently become possible using novel adenoviruses, OBP-301 (4–6) and, more recently, OBP-401 (7, 8). This latter virus, which can enter most cells, contains the replication cassette with the human telomerase reverse transcriptase (hTERT) promoter driving the expression of the viral E1 genes, and the inserted GFP gene. Virus replication and, hence, GFP gene expression occur only in the presence of an active telomerase, i.e., in malignant tissue (7). The OBP-401 virus was first tested by injection directly into HT-29 human colon tumors orthotopically implanted into the rectum in BALB/c nu/nu mice (7). Subsequent para-aortic lymph node metastasis was observed by laparotomy under fluorescence. The adaption of GFP fluorescence to in vivo labeling of tumor tissue should facilitate precision surgical navigation in live animals and, very possibly, in a clinical surgical setting.

Results

Fluorescence Labeling of Human Cancer Cells with OBP-401 in Vitro. A549 tumor cells, growing in tissue culture, were infected with OBP-401, and the development of GFP fluorescence followed. The fluorescence intensity gradually increased after infection as the virus, with its GFP gene, replicated (Fig. 1 A).

The extent of infection was tested by infecting red fluorescent protein (RFP)-expressing cancer cells, growing in cell culture, with OBP-401. These included A549-RFP, PC-3-RFP, HCT-116-RFP, and HT-29-RFP cells. In most cells, the introduction of green fluorescence changes the cell color from red to yellow, showing that most were infected by OBP-401. Any remaining red fluorescence clearly identifies those few cells that remain uninfected by the adenovirus. The color changes increased gradually followed by cell death due to the cytopathic effect of replicating OBP-401 (Fig. 1 B and C).

Fluorescence Labeling of Subcutaneous Tumors by Infection in Vivo with OBP-401. Both nonfluorescent PC-3 and red fluorescent PC-3-RFP human prostate cancer cells were inoculated s.c. (Fig. 2 A and B). The resulting s.c. tumors were injected with 1 × 10⁶ plaque-forming units (PFU) of OBP-401 as shown in Fig. 2 B. A color change from red to yellow in the s.c. PC-3-RFP tumor and the onset of GFP fluorescence in the nonfluorescent PC-3 tumor were observed by the third day after virus injection (Fig. 2 C). An RFP filter selectively showed the tumors’ endogenous RFP fluorescence (Fig. 2 D). Similarly, a GFP filter showed GFP fluorescence induced in the tumors by OBP-401 (Fig. 2 E). Infecting tumor cells that are endogenously expressing RFP with the GFP-expressing adenoviral vector OBP-401 clearly shows the extent of GFP labeling of the tumor. Cells showing a yellow fluorescence are infected with OBP-401, while the remaining red fluorescent cells clearly indicate the small portion that might remain uninfected.

Labeling Peritoneal Carcinomatosis with OBP-401. Peritoneal carcinomatosis was induced in the abdominal cavity of nude mice by inoculating 3 × 10⁶ red fluorescent HCT-116-RFP human colorectal cancer cells. Various sized peritoneal disseminated nodules developed within 12 days. These were clearly visible by fluorescence imaging using a long-pass filter and/or a specific RFP filter (Fig. 3 A and B). Even very small disseminated nodules were illuminated by RFP fluorescence (Fig. 3 B). Although there was some autofluorescence from adjacent organs visible, the tumor nodules were not visible through a GFP filter (Fig. 3 A and B).

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1To whom correspondence may be addressed. E-mail: penman@mit.edu or all@anticancer.com.

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Once the malignant nodules were established at 12 days after intraperitoneal (i.p.) implantation of HCT-116-RFP cells, \(1 \times 10^8\) PFU OBP-401 were injected into the mouse abdominal cavity. Selective color filters showed that the HCT-116-RFP disseminated nodules expressed GFP fluorescence as well as RFP when examined 5 days later (Fig. 3C). RFP fluorescence...
was essentially coincident with that of GFP (Fig. 3C). These results indicate that i.p. injection of OBP-401 efficiently infected and labeled disseminated cancer.

Labeling of Pleurally Disseminated Cancer with OBP-401. These experiments assessed the effectiveness of OBP-401 labeling of pleural carcinomatosis in a mouse model of unlabeled A549 human lung cancer cells. The thoracic space of nude mice was inoculated with $2 \times 10^6$ cancer cells. Various sized disseminated plural nodules appeared within 10 days after implantation. At this time, $1 \times 10^8$ PFU of OBP-401 were injected into the thoracic cavity. Five days after injection of OBP-401, the cavity was examined using GFP fluorescence imaging. A representative mouse is shown in Fig. 4. Disseminated pleural nodules were visualized by GFP expression (Fig. 4A and B). Even very small lesions, which are normally undetectable, were clearly illuminated by GFP fluorescence (Fig. 4C). Histological examination confirmed that these GFP-expressing tissues were adenocarcinomas. A representative histological section is shown in Fig. 4D. These results suggest that intrapleural injection of at least $1 \times 10^8$ PFU of OBP-401 can efficiently label disseminated pleural cancer. Lower doses of OBP-401 resulted in less efficient labeling.

OBP-401 Fluorescence-Guided Resection of Disseminated Peritoneal Tumors. In order to test the effectiveness of OBP-401-guided cytoreduction surgery, we used the peritoneal carcinomatosis model with nonfluorescent HCT-116 human colon cancer cells. Mice with peritoneal carcinomatosis were injected i.p. with OBP-401 at a dose of $1 \times 10^8$ PFU. Five days after viral administration, laparotomy was performed to remove intra-abdominal disease using fluorescence-guided navigation under anesthesia (Fig. 5A and B). A representative mouse after cytoreduction surgery with OBP-401-navigation is shown in Fig. 5C. Disseminated cancer nodules, which would otherwise be undetectable, were clearly visible by bright GFP fluorescence. The resected nodules were visualized as frozen sections under both fluorescence (Fig. 5D) and after hematoxylin and eosin (H&E) staining (Fig. 5E and F). These results suggest that OBP-401-labeling has significant potential for guiding cytoreduction surgery of disseminated cancer.
Discussion
The peritoneal surface is involved in more than 20% of patients with gastric, colon, and pancreatic cancers (1). Cytoreduction surgery requires resection of all visible tumors and stripping of all peritoneal surfaces that contain metastatic nodules (1, 2, 9). Therefore, visceral peritoneal involvement often requires concomitant resection of intra-abdominal organs such as the small intestine and colon.

The detection of very small peritoneal lesions is largely limited by the weak contrast between tumor nodules and surrounding normal tissues. Technology improving the intraoperative detection of peritoneal disease would facilitate essentially complete cytoreduction in these patients. The photosensitizer, 5-aminolevulinic acid (5-ALA) has been used for intraoperative detection of cancer lesions in neurosurgery (10). However, labeling that is essentially cancer-selective can be a powerful surgical adjunct. This report shows that OBP-401 infection of cancer cells leads to the highly selective induction of bright GFP fluorescence.

Implanting RFP-expressing cancer cell lines gave rise to fluorescent nodules whose color change clearly indicated the efficiency with which OBP-401 labeled disseminated peritoneal tumors with GFP. The change from red to yellow fluorescence indicated successful infection by OBP-401 (Fig. 3). Similarly, OBP-401 GFP labeling could detect dissemination nodules with high sensitivity in a pleural carcinomatosis model (Fig. 4).

Perhaps most importantly, we could remove disseminated disease in a peritoneal carcinomatosis model by using fluorescence-guided resection. These results suggest developing a dedicated excitation for fluorescence-guided surgery similar to that described for use in mice (11). In the present study, during surgery, even very small peritoneal lesions could be identified with GFP fluorescence (11).

Materials and Methods

Recombinant Adenovirus. OBP-401, containing the GFP gene under the control of the CMV promoter with the hTERT promoter driving the E1A and E1B genes, was constructed as previously described (6, 7). OBP-401 was purified by ultracentrifugation in cesium chloride step gradients. Virus titers were determined by a plaque-forming assay using 293 cells. The virus was stored at –80°C.

Cell Culture. The human non-small cell lung cancer cell line A549, the human colorectal cancer cell lines HCT-116 and HT-29, and the human prostate cancer cell line PC-3 were cultured in RPMI 1640 medium supplemented with 10% FBS.

Production of RFP Retroviral Vector. For RFP retrovirus production, the HindIII/Not fragment from pDsRed2 (Clontech), containing the full-length RFP cDNA, was inserted into the HindIII/Not site of pLNCX2 (Clontech) containing the neomycin-resistance gene. PT67, a NIH 3T3-derived packaging cell line (Clontech), expressing the viral envelope, was cultured in DMEM supplemented with 10% FBS. For vector production, PT67 packaging cells, at 70% confluence, were amplified by conventional culture methods in the absence of selective agent. The supernatant was collected and the cells were harvested with trypsin/EDTA 18 h after this time. The viral supernatant was concentrated at 50°C, filtered, and stored at –80°C.

Fluorescence Optical Imaging and Processing. An Olympus OV100 Small Animal Imaging System containing an MT-20 light source was used. High-resolution images were captured directly on a PC (Fujitsu Siemens). Images were analyzed with the use of Cell® software (Olympus Biosystems) (16).

Histological Examination. For histological studies, GFP-expressing tissues were removed and examined microscopically.

Fluorescence Imaging of Tumor-Host Interactions. A mouse was anesthetized with an inhalation agent (isoflurane) and the abdominal cavity was opened. An LED flashlight (Olympus) was used to excite the area and GFP fluorescence was visualized through an optical filter (H11002) with an emission wavelength of 535 nm. Images were captured on a PC (Fujitsu Siemens). Images were analyzed with the use of Cell® software (Olympus Biosystems) (16).

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