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IN VIVO VISUALIZATION OF EPIDERMAL GROWTH FACTOR RECEPTOR AND SURVIVIN EXPRESSION IN PORCINE PANCREAS USING ENDOSCOPIC ULTRASOUND GUIDED FINE NEEDLE IMAGING WITH CONFOCAL LASER-INDUCED ENDOMICROSCOPY

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The aims of this pilot study were to establish a principle of molecular imaging of the pancreas and determine *in vivo* expression of epidermal growth factor receptor (EGF-R) and survivin using a novel endoscopic ultrasound-guided fine needle imaging (EUS-FNI) technique, which incorporates needle based confocal laser-induced endomicroscopy (nCLE) after intrapancreatic injection of FTIC-labeled antibodies. Studies were performed in anesthetized pigs. FITC-labeled specific antibodies against EGF-R and survivin were injected into the tail and neck of the pancreas using a 19 gauge needle introduced under EUS guidance. Thirty minutes later, nCLE was performed using a prototype needle-based confocal laser-induced endomicroscopy probe (Cellvizio AQ-Flex-19, Mauna Kea Technologies, Paris, France) to determine cellular and tissue localization of EGF-R and survivin in the pancreas. Then pigs were euthanized and specimens of pancreas from areas injected with antibodies were obtained for histologic examination under epifluorescence microscope. Results: EUS-guided nCLE enabled visualization of EGF-R and survivin in pancreatic tissue. Expression of EGF-R and survivin in pancreas was confirmed by histology. EGF-R immunoreactivity was localized to majority of duct-lining cells and to the surface and cytoplasm of many acinar cells. Survivin was localized mainly to the acinar cells. This study demonstrated the feasibility of *in vivo*, real time visualization of EGF-R and survivin in the pancreas by local injection of FITC-labeled antibodies *via* EUS-guided fine needle injection, followed by EUS-guided needle based confocal laser-induced endomicroscopy.

Key words: *confocal laser-induced endomicroscopy, epidermal growth factor receptor, survivin, pancreas, molecular imaging*

INTRODUCTION

In our previous publication we reported a successful *in vivo* molecular imaging of epidermal growth factor receptor (EGF-R) and survivin in esophageal and gastric mucosa in pigs using probe-based confocal laser-induced endomicroscopy (pCLE) and topical application of FITC-labeled specific antibodies (1). In that paper we also elaborated on the pCLE technique, which allows for non-invasive, *in vivo*, real time visualization of gastrointestinal mucosal tissue and cell structures under 1,000× magnification during endoscopy (2-5) and for *in vivo* assessment of mucosal functions, *e.g.* mucosal permeability (6). The imaging of esophageal and gastrointestinal mucosa was performed using pCLE through an endoscope (1). The luminal CLE probes introduced *via* the instrumentation channel of a standard endoscope are predominantly designed for gastrointestinal mucosal application (3-6). The imaging depth is probe dependent and limited to 30 to 150 μm, which makes the imaging of intra-abdominal organs, such as pancreas impossible. A new prototype for needle-based confocal laser-induced endomicroscopy (nCLE) probe (Cellvizio AQ-Flex, Mauna Kea Technologies, Paris, France) has been recently developed. This prototype is flexible with a diameter of 0.77 mm and can be introduced through a standard 19-gauge needle, used for endoscopic ultrasound (EUS)-guided fine needle aspiration (FNA).

Previous studies demonstrated that EGF-R is expressed in normal pancreas in ductal and acinar cells, where it plays regulatory role in pancreatic exocrine function (7-11). EGF-R mediates EGF-induced potentiation of secretagogue activated release of digestive enzymes (8), pancreatic acinar cell survival in serum free cultures (9) and trophic and growth promoting action on pancreatic tissue (10, 11). The expression of EGF-R is significantly upregulated in pancreatic cancer (7).

The expression of survivin (an anti-apoptosis protein) in the pancreas has been predominantly reported in the context of pancreatic cancer. Survivin is strongly upregulated in pancreatic cancer and likely promotes cancer cell growth by preventing apoptosis and by promoting mitosis (12, 13). The expression of survivin and its role in normal pancreas has not been fully explored; one study demonstrated that survivin expression in the pancreatic islets during perinatal remodeling is crucial for the development of beta cell mass (14). Another study showed that survivin mRNA and protein are upregulated 36 hours after

induction of experimental pancreatitis and suggested survivin's role in cell protection and pancreatic regeneration (15). In this regard survivin's role in pancreas is similar to that in gastric epithelium (16). A recent study demonstrated that EGF (*via* EGF-R) upregulates survivin expression in pancreatic beta cells during the neonatal period (17), thus indicating a possibility of local autocrine and/or paracrine regulation. Therefore, visualization of EGF-R and survivin expression *in vivo* in the pancreas is important for a better understanding of their pathophysiological roles.

In the present study we examined the feasibility of *in vivo*, real time visualization of EGF-R and survivin in the pancreas by local injection of FITC-labeled antibodies *via* EUS-guided fine needle injection, followed by EUS-guided needle based confocal laser-induced endomicroscopy.

MATERIAL AND METHODS

Experimental design

This study was aimed to establish a new paradigm and to perform *in vivo* labeling and imaging of EGF-R and survivin in the pancreas. This is more complex than CLE imaging of gastrointestinal mucosa and requires EUS guided administration of FITC-labeled antibodies against EGF-R and survivin using a FNA needle into two different regions of the pancreas. Thirty minutes later an nCLE probe was inserted under EUS guidance to the pancreatic tail and neck *via* a 19 gauge needle into the same areas to perform imaging of EGF-R and survivin expression.

Two pigs were examined under general anesthesia with endotracheal intubation and controlled ventilation in the Comprehensive Digestive Disease Center animal lab. Pre-anesthesia sedation was provided with an intramuscular injection of azaperone (2.0 mg/kg), ketamine (10 mg/kg), and atropine (0.02 mg/kg). Anesthesia was performed with a continuous infusion of pentobarbitone 25–35 mg/kg/h. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

EUS was performed using an electronic linear scanning video echoendoscopes (GF-UCT140-AL5; Olympus America Inc., Canter Valley, PA, USA) in combination with a processor (Pro-sound Alpha 5; Aloka America Inc., Wallingford, CT, USA). nCLE visualization of pancreas was performed using a prototype probe (AQ-Flex, Cellvizio, Mauna Kea Technologies, Paris, France) through a 19 gauge FNA needle (Echotip Ultra Endoscopic Ultrasound Needle, ECHO-19, Cook Medical, Bloomington, IN, USA). A prototype AQ-Flex probe has a diameter of 0.85 mm, a field view of 325 µm with a resolution of 3.5 µm and a focal depth of 40–70 µm.

Antibodies

Anti-human EGFR-fluorescein conjugated monoclonal antibody (FAB10951F; 1:100 in PBS supplemented with 0.5% BSA) and anti-human survivin-fluorescein conjugated monoclonal antibody (IC6472F; 1:100 in HBSS supplemented with 0.1% saponin) from R&D Systems, Minneapolis, MN) were used. Saponin in a balanced salt solution is effective in facilitating antibody entry into the cells. The antibodies, diluted 1:100 were injected under EUS guidance *via* FNA needle into pancreatic tail and neck areas. Thirty minutes after antibodies injection, a prototype nCLE probe (Cellvizio AQ-Flex, Mauna Kea Technologies, Paris, France) was inserted into pancreas *via* 19 gauge FNA needle under EUS guidance and nCLE determination of EGF-R and survivin expression was performed.

At the end of experiments pigs were euthanized using a lethal dose of pentobarbitone and pancreatic sections from the tail and neck areas injected with antibodies were obtained, fixed in 10% buffered formalin and routinely processed for histology. Five µm thin sections were deparaffinized and examined under a Nikon fluorescence microscope.

RESULTS

Needle-based confocal laser-induced endomicroscopy images

Control images were obtained with EUS-guided nCLE within the pancreas without any injection. These images showed no fluorescent structures in the pancreas.

After injection of fluorescein labeled anti-EGF-R antibody, EUS-guided nCLE revealed thick and irregular inter-connected bright strands (*Fig. 1*).

After injection of fluorescein labeled anti-survivin antibody, EUS-guided nCLE revealed a diffuse ground-glass background with thin and ultrathin bright strands with occasional branching (*Fig. 2*).

Histologic data

EGF-R and survivin were expressed in pancreatic tissue. EGF-R was localized predominantly to the majority of ductal

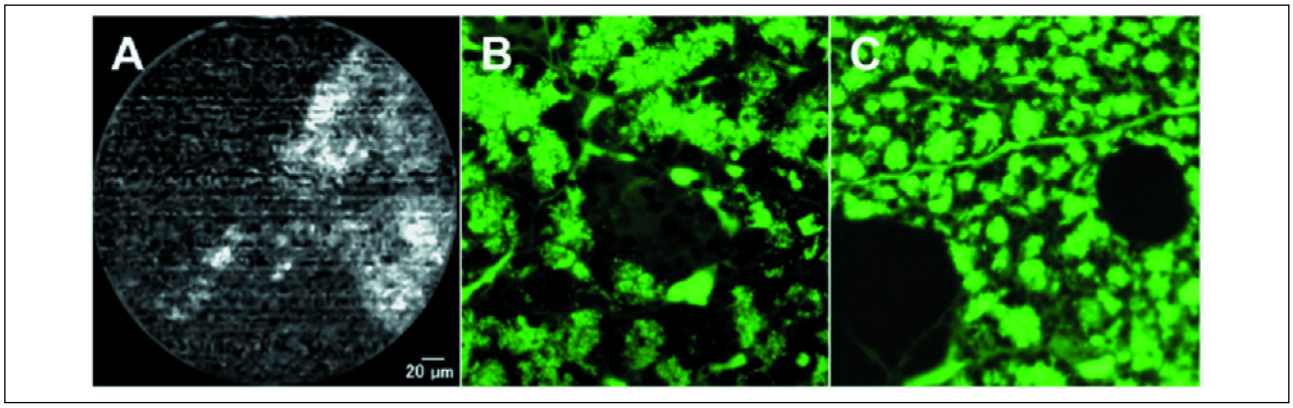

Fig. 1. nCLE and histologic images of pancreas injected with fluorescence-labeled anti-EGF-R antibody. (A): nCLE image after injection of fluorescein labeled anti-EGF-R antibody, showing thick and irregular inter-connected bright strands. (B) and (C): Micrographs of histologic sections of pancreas showing EGF-R protein localized predominantly to the majority of duct-lining cells and to the surface and cytoplasm of numerous acinar cells.

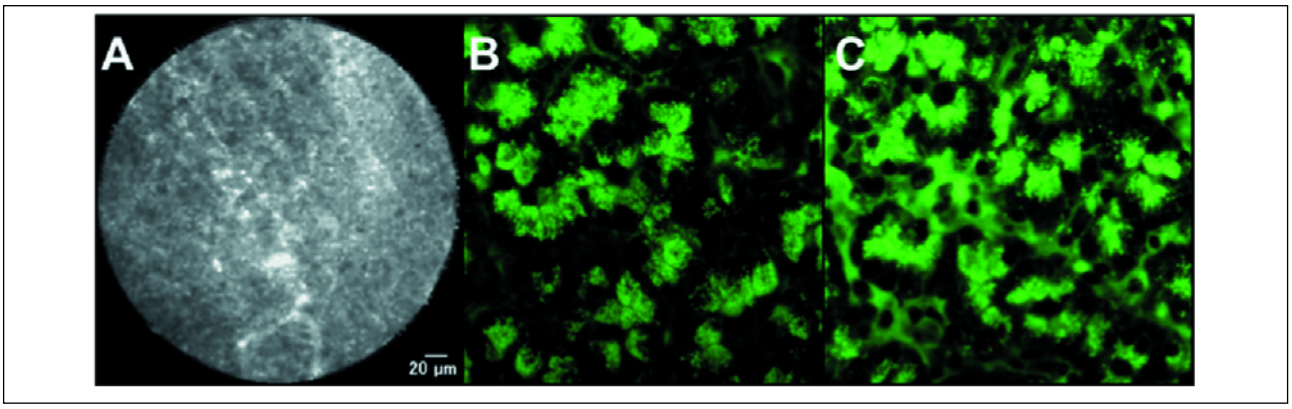

Fig. 2. nCLE and histologic images of pancreas injected with fluorescence-labeled anti-survivin antibody. (A): nCLE image after injection of fluorescein labeled anti-survivin antibody showing a diffuse ground-glass background with thin and ultrathin bright strands with occasional branching. (B) and (C): Micrographs of histological sections of pancreas showing survivin localized mainly to the acinar cells.

cells and to the surface and cytoplasm of numerous acinar cells. Survivin was localized mainly to the acinar cells.

DISCUSSION

This study demonstrated feasibility of *in vivo*, real time visualization of EGF-R and survivin in the pancreas using needle-based CLE in combination with EUS-FNA and injection of FITC-labeled antibodies without necessity of laparotomy. It demonstrated for the first time a successful *in vivo* visualization of EGF-R and survivin in porcine pancreas using a needle-based CLE probe, EUS-FNA and FITC- labeled specific antibodies. From the technical point of view such study requires an advanced expertise in both CLE imaging and EUS-FNA (18).

This study established a novel method and a paradigm of molecular imaging of pancreas, which has important implications. Needle-based CLE under EUS-FNA guidance allows *in vivo* visualization of specific regulatory protein and receptors in pancreas, that potentially have important implications for cell growth, proliferation and apoptosis. Moreover, by using antibodies against phosphorylated EGF-R, this procedure will make it possible to determine *in vivo*, non-invasively the state of receptor phosphorylation/activation and its response to physiological and pharmacological stimuli. Once this method is optimized it will allow quantification of expression of these proteins in a similar way as in our previous *ex-vivo* study (6).

Previously, Fottner *et al*. successfully performed *in vivo* molecular imaging of somatostatin receptors in pancreatic islet cells and neuroendocrine tumors using miniaturized confocal laser-scanning fluorescence microscopy and fluorescein-labeled octreotate in healthy mice and murine models of neuroendocrine tumors (19). However, the visualization and imaging of mice pancreas in that study required laparotomy and the use of hand-held probe, which cannot be used in human CLE (19).

Recent pioneering studies using a needle-based CLE probe established a technical feasibility of this method to visualize pancreas in porcine models and in humans (20, 21). However, none of these studies attempted *in vivo* molecular imaging of important regulatory proteins such as EGF-R and survivin in the pancreas.

In addition to visualization of cellular and tissue structures needle-based CLE enables to study *in vivo* pathophysiological events in natural tissue environment, and hence functional imaging. *In vivo* molecular imaging with needle-based CLE can be used in basic science and clinical setting and will enable better understanding of pancreatic pathophysiology.

Conflict of interests: None declared.

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