JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2012, 63, 3, 303-307
www.jpp.krakow.pl

Short communication

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MOLECULAR IMAGING OF EPIDERMAL GROWTH FACTOR-RECEPTOR AND SURVIVIN *IN VIVO* IN PORCINE ESOPHAGEAL AND GASTRIC MUCOSAE USING PROBE-BASED CONFOCAL LASER-INDUCED ENDOMICROSCOPY: PROOF OF CONCEPT

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Confocal laser-induced endomicroscopy (CLE) enables *in vivo*, real time visualization of the subsurface cells and tissue structures in gastrointestinal mucosa at a subcellular resolution of ~1000× magnification. The aims of this pilot study were to establish a principle of molecular imaging and determine *in vivo* expression of epidermal growth factor receptor (EGF-R) and survivin in porcine esophageal and gastric mucosa using probe-based CLE (pCLE) and topically applied FITC-labeled antibodies. Studies were performed in anesthetized pigs. During endoscopy FITC-labeled antibodies against EGF-R and survivin were either sprayed onto esophageal and gastric mucosa in preselected areas or administered *via* submucosal injection. Thirty minutes later pCLE was performed using a through-the-scope probe (GastroFlex UHD, Cellvizio, Mauna Kea Technologies, Paris, France) to determine cellular and tissue localization of EGF-R and survivin. Then the pigs were euthanized and esophageal and gastric walls from the areas sprayed or injected with antibodies were collected for histologic examination under epifluorescence microscopy. Results: CLE enabled visualization of EGF-R and survivin in esophageal and gastric mucosa and this was confirmed by histology. In the esophagus both EGF-R and survivin were localized predominantly to the keratinocyte progenitor cells. In the stomach, EGF-R was localized to progenitor zone cells and some epithelial cells. Localization of survivin was similar, but involved more surface epithelial cells. This study demonstrated feasibility of using CLE and topical administration of FITC labeled antibodies for *in vivo* localization of EGF-R and survivin in esophageal and gastric mucosa.

Key words: *confocal laser endomicroscopy, epidermal growth factor-receptor, esophageal mucosa, gastric mucosa, molecular imaging, survivin*

INTRODUCTION

Recent development of powerful, confocal laser endomicroscopic (CLE) imaging systems (Pentax, OptiScan 5 and CellVizio) now allows non-invasive, *in vivo*, real time visualization and detailed evaluation of tissues, vasculature, cell structures under 1000× magnification (1-7). CLE allows visualization of the surface epithelium, subepithelial glandular structures, and mucosal microvasculature. Moreover, probe-based CLE (pCLE) allows *in vivo*, non-invasive, real time assessment of mucosal functions, *e.g.* erythrocyte flow velocity and mucosal permeability as demonstrated in a recent paper (8). Some recent studies demonstrated that CLE could be used for molecular imaging (9, 10). For example, molecular imaging of vascular endothelial growth factor (VEGF) was performed by CLE using labeled antibodies in tumors in APC min mice, in xenograft models and in surgical specimens of patients with colorectal cancer and these findings were correlated with *ex vivo* microscopy (10). In a recent study, *in vivo* pCLE imaging was performed to determine vascular permeability and visualize vascular structures in colonic polyps (8). It was combined with detection of VEGF expression on histolologic biopsies obtained during CLE procedure (8).

Epidermal growth factor receptor (EGF-R) is expressed in esophageal and gastric mucosa (11). Immunohistochemical staining of gastric sections demonstrated that in the stomach EGF-R is predominantly localized to epithelial cells and their progenitors (11) and is critical for continual mucosal renewal and thus for maintenance of epithelial structures' integrity and functions. Our previous studies demonstrated the critical role of EGF-R and EGF-R/RAS/MAPK signaling pathway in gastric ulcer healing (12, 13). Moreover, molecular biology studies demonstrated that EGF-R is transactivated by prostaglandin E_2 (PGE_2) and this mechanism underlies mucosal growth-promoting action of PGE_2 (14).

Survivin, a 16.5-kilodalton protein, is a member of the inhibitors of apoptosis protein family, broad-spectrum suppressor of cell death and a protein promoting mitosis (15-19). Initially it was discovered as a cancer promoting protein, since it allows cancer cells to avoid apoptosis (15, 16). Survivin inhibits apoptosis by binding to caspase-3 and caspase-7 and also inhibits caspase-independent cell death (17-19). Previous studies showed that survivin is expressed in normal gastric mucosa of adult humans and rats, predominantly in the epithelial progenitor cells and also in mucosal surface epithelial cells, which are

directly exposed to damaging agents such as acid and NSAIDs (18, 20). Furthermore, a recent study demonstrated that survivin is expressed at much higher levels in cultured gastric mucosal epithelial cells than in gastric microvascular endothelial cells (21); and, that this difference in expression is similar to the expression pattern found in intact gastric tissues (21). The expression pattern of survivin within the gastric mucosa, together with survivin's known anti-apoptosis function, suggests that survivin plays crucial role in maintaining gastric mucosal integrity and protection of the gastric mucosa against injury (17-20). Survivin is a major target for mucosal injury by a non-selective NSAIDs and reduction of survivin predisposes gastric mucosal cells to a greater severity of injury (18). Furthermore, another study showed that survivin is a key mediator of adaptive cytoprotection against ethanol-induced gastric injury (22).

This present study was aimed to establish a principle of molecular imaging and to determine *in vivo* expression of epidermal growth factor receptor (EGF-R) and survivin in esophageal and gastric mucosa in pigs using pCLE and topically applied FITC-labeled antibodies. We used pCLE fiber optic probe with a diameter 2.6 mm for non-invasive assessment of expression of epidermal growth factor receptor (EGF-R) and survivin in esophageal and gastric mucosa of pigs using FITC-labeled specific antibodies. The Cellvizio Endomicroscopy System is based on a catheter probe with a semiconductor safe, class 2, photodiode laser that oscillates at 488 nm and is sensitive down to single pixel detection. The Cellvizio confocal miniprobes for GI tract applications generate high resolution (scanning field of 30,000 pixels) images at 12 frames per second, which are reconstructed by a special computer algorithm allowing dynamic assessment of GI tissue structure and vasculature once the single video frames are in an image with an enlarged field of view (4 mm×2 mm) (5).

EXPERIMENTAL DESIGN AND METHODS

The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of California, Irvine. Two pigs were examined under general anesthesia with endotracheal intubation and controlled ventilation at the animal lab of UCI medical center. Preanesthesia sedation was given 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.

CLE visualization of esophageal and gastric mucosa was performed using the ultra high-definition (UHD) probe (GastroFlex UHD, Cellvizio, Mauna Kea Technologies, Paris, France) through a high-resolution endoscope (GIF-H180; Olympus America, Center Valley, PA). GastroFlex UHD probe has a penetration depth of 60 µm, field of view of 240 µm, a resolution of 1 µm, and a magnification of ×1,000.

Labeled antibody, dilution, application

Anti-human EGF-R-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. Antibodies diluted 1:100 were sprayed onto the esophageal and gastric antral mucosa using a spray catheter (PW-5L-1, Olympus America) and in separate experiments injected into esophageal and gastric fundic submucosa using an injection needle (LDVI-23-240, Cook Medical, Bloomington, IN, USA. Thirty minutes after topical application of antibody, pCLE imaging of the esophageal and gastric mucosa was performed.

At the end of experiments, the animals were euthanized using a lethal dose of pentobarbitone. Esophageal and gastric mucosal sections were obtained from the areas sprayed or injected, fixed in 10% buffered formalin and routinely processed for histology. Five µm thin sections were deparaffinized and examined under a Nikon florescence microscope.

RESULTS

Probe-based confocal laser-induced endomicroscopy images

In the esophagus, pCLE, using spray and submucosal injection of fluorescein antibody, demonstrated uptake in bright "lakes" (20 to 40 microns), and fine linear strands (5 to 10 microns), in a background of very faint uptake and black dots, which likely represent cell nuclei. Bright "lakes" and strands

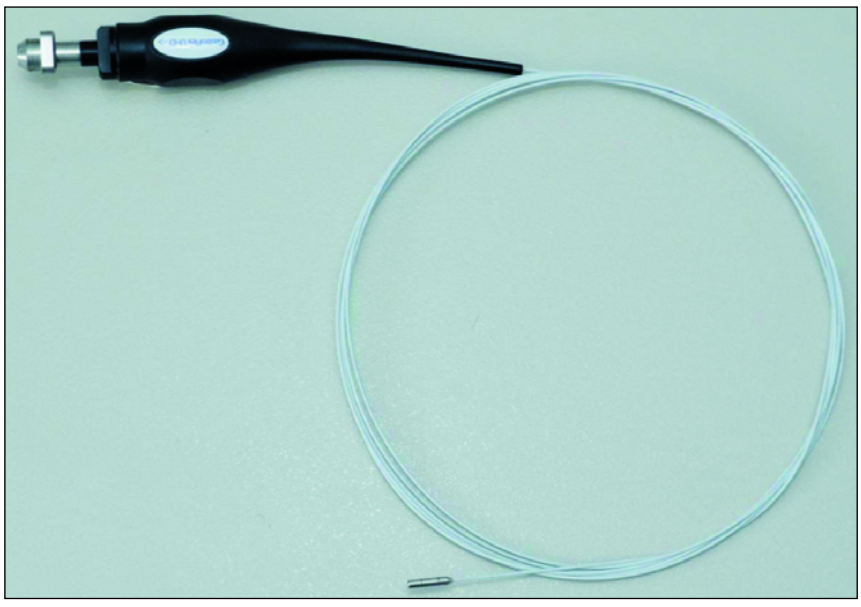

Fig. 1. CLE probe-GastroFlex UHD, Cellvizio, Mauna Kea Technologies, Paris, France used for probe-based confocal-laser endomicroscopy. This probe is introduced through a biopsy channel of an endoscope.

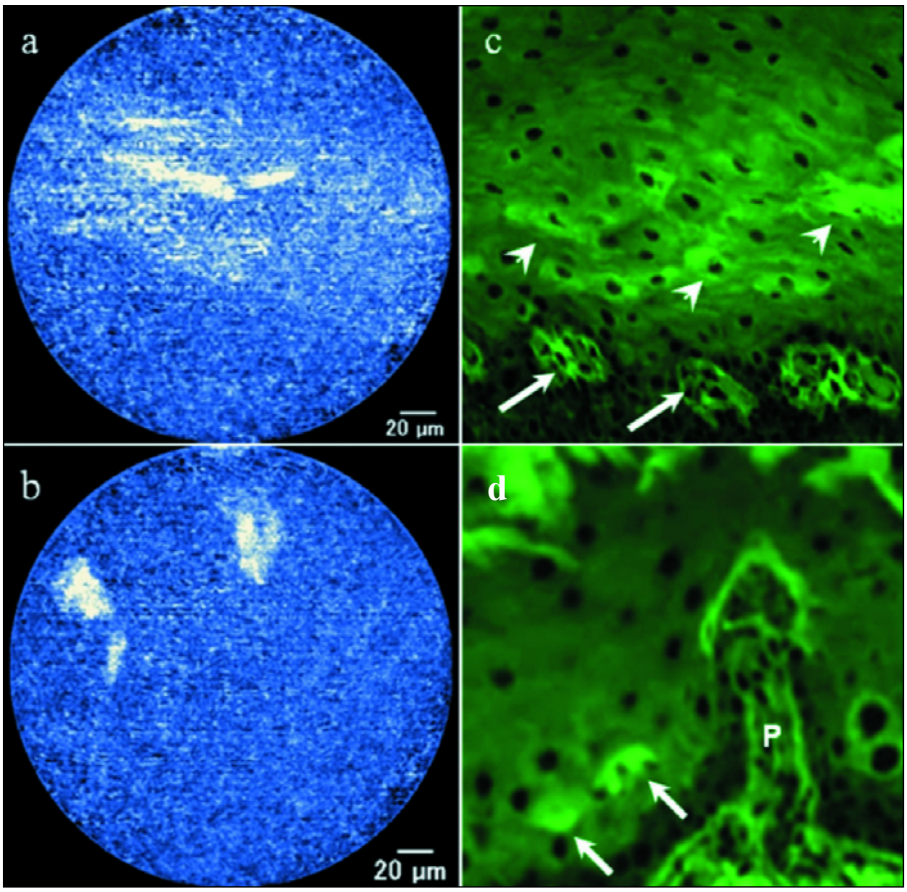

Fig. 2. pCLE and histologic images of esophagus with anti-EGF-R antibody. (a) pCLE after spray, and (b) pCLE after submucosal injection showed fluorescein uptake in bright "lakes" and fine linear strands. (c) and (d) micrographs of esophageal mucosa. Fluorescence is localized to keratinocyte progenitor cells (arrows) and to some keratinocytes of stratum spinosum (arrowheads); P -papilla.

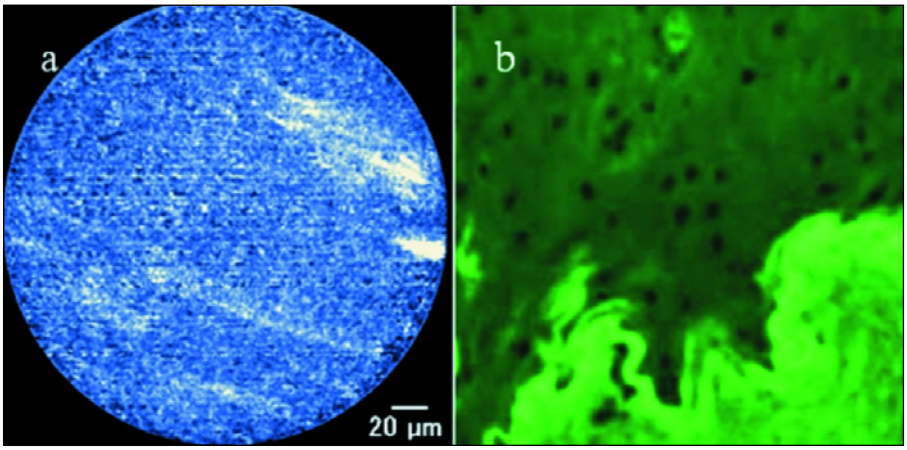

Fig. 3. pCLE and histologic images of esophagus with anti-survivin antibody. (a) pCLE after spray showed fluorescein uptake in bright "lakes" and fine linear strands. (b) Micrograph of esophageal mucosa. Fluorescence is localized to basal mucosal layer.

were found on both anti-EGF-R antibody (*Fig. 2*) and anti-survivin antibody (*Fig. 3*) images.

In the stomach using anti-EGF-R antibody, glandular structures with nuclei located on the basal part of the cells were present in the antrum and thick and irregular strands in the fundus (*Fig. 4*). With anti-survivin antibody, bright "lakes" and strands were more pronounced in the antrum than in the fundus (*Fig. 5*).

Histologic data

In esophageal squamous epithelial mucosa, both EGF-R (*Fig. 2*) and survivin (*Fig. 3*) were localized predominantly to the keratinocyte progenitor cells localized in the basal mucosal layer. In the stomach, EGF-R was localized to progenitor zone cells in the neck area of gastric glands and to some epithelial cells (*Fig. 4*). Survivin expression in gastric mucosa was mainly localized to the progenitor cells, often with nuclear localization, and also to the surface epithelial cells (*Fig. 5*).

DISCUSSION

This is the first successful *in vivo* visualization of EGF-R and survivin in esophageal and gastric mucosa in pigs using probe-based confocal laser endomicroscopy and FITC-labeled specific antibodies. This study established a novel method and a paradigm, which has important basic science and clinical implications. pCLE allows for real time, *in vivo* determination of the distribution of specific regulatory proteins and receptors in the GI tract mucosa, which have important implications for cell

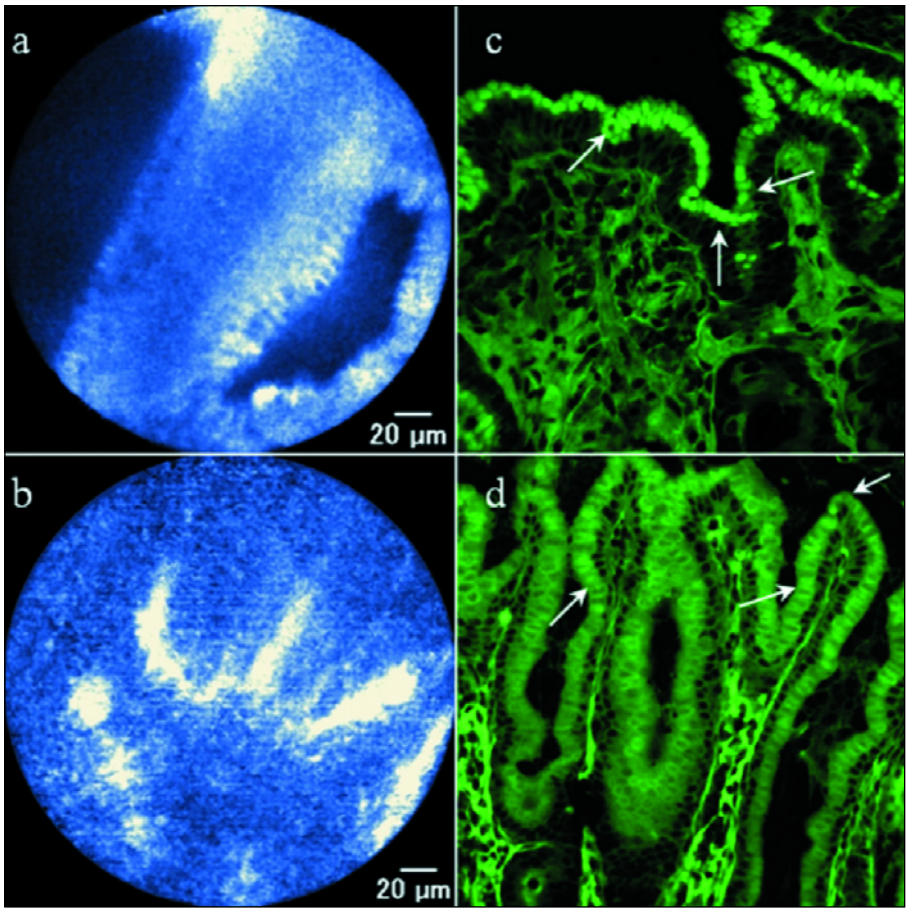

Fig. 4. pCLE and histological images of stomach with anti-EGF-R antibody. (a) pCLE after spray in the antrum showed glandular structures lined with epithelial cells and (b) pCLE after submucosal injection in the fundus showed thick and irregular strands. (c) and (d) micrographs of gastric mucosa. Fluorescence is localized to progenitor zone cells and some surface epithelial cells (arrows).

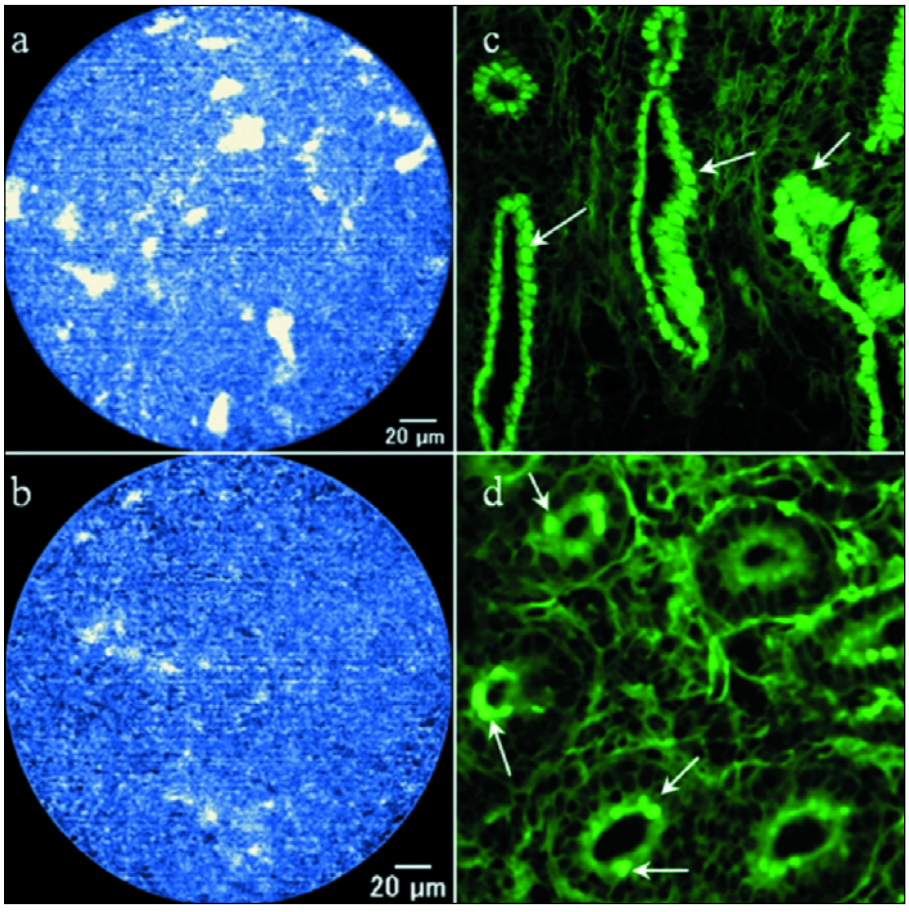

Fig. 5. pCLE and histologic images of stomach with anti-survivin antibody. (a) pCLE after spraying in the antrum and (b) pCLE after submucosal injection in the fundus showed bright “lakes” and strands. (c) and (d) micrographs of gastric mucosa. Fluorescence is localized to progenitor zone cells, often to the nuclei (d; arrows) and numerous surface epithelial cells (c; arrows).

growth, proliferation and apoptosis. In addition, pCLE offers *in vivo* visualization, and monitoring of cellular structure and functions over time. The sequential assessment structural changes provides new insights into dynamic morphology and physiology. Moreover, by using in future studies antibodies against phosphorylated EGF-R it may be possible to determine *in vivo*, non-invasively, the state of receptor activation and its response to physiological and pharmacological stimuli. In addition to visualization of cellular and tissue structures CLE provides an opportunity to study *in vivo* pathophysiological events in natural tissue environment, and hence functional imaging. *In vivo* molecular imaging with CLE can be used in basic science and clinical setting and will enable better understanding of gastrointestinal pathophysiology.

Acknowledgments: This research was supported by H.H. Chao Comprehensive Digestive Disease Center, University of California, Irvine (KJC) and in part by VA Merit Review (AST).

Conflict of interests: None declared.

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Received: May 10, 2012
Accepted: June 20, 2012

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