Optical Biopsy of Human Bladder Neoplasia With In Vivo Confocal Laser Endomicroscopy

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Purpose: Confocal laser endomicroscopy is a new endoscopic imaging technology that could complement white light cystoscopy by providing in vivo bladder histopathology. We evaluated confocal laser endomicroscopy by imaging normal, malignant appearing and indeterminate bladder mucosa in a pilot study.

Materials and Methods: Patients scheduled to undergo transurethral resection of bladder tumors were recruited during a 3-month period. After standard cystoscopy fluorescein was administered intravesically and/or intravenously as a contrast dye. A 2.6 mm probe based confocal laser endomicroscope was passed through a 26Fr resectoscope to image normal and abnormal appearing areas. The images were collected with 488 nm excitation at 8 to 12 frames per second. The endomicroscopic images were compared with standard hematoxylin and eosin analysis of transurethral resection of bladder tumor specimens.

Results: Of the 27 recruited patients 8 had no cancer, 9 had low grade tumors, 9 had high grade tumors and 1 had a low grade tumor with a high grade focus. Endomicroscopic images demonstrated clear differences between normal mucosa, and low and high grade tumors. In normal urothelium larger umbrella cells are seen most superficially followed by smaller intermediate cells and the less cellular lamina propria. In contrast, low grade papillary tumors demonstrate densely arranged but normal-shaped small cells extending outward from fibrovascular cores. High grade tumors show markedly irregular architecture and cellular pleomorphism.

Conclusions: We report the first study to our knowledge of in vivo confocal laser endomicroscopy in the urinary tract. Marked differences among normal urothelium, low grade tumors and high grade tumors were visualized. Pending further clinical investigation and technological improvement, confocal laser endomicroscopy may become a useful adjunct to conventional cystoscopy.

Key Words: microscopy, confocal; urinary bladder neoplasms; anatomy and histology; biopsy

Abbreviations and Acronyms
TURBT = transurethral resection of bladder tumor

DIAGNOSTIC cystoscopy is an integral part of urinary tract evaluation, particularly for bladder cancer. Nevertheless, numerous well recognized limitations include operator dependency, differentiation of inflamed mucosa from flat neoplastic lesions and lack of real-time tumor grade or stage information. Procedures to obtain tissue diagnosis including TURBT and random bladder biopsy carry risks of anesthesia, bleeding and bladder perforation in addition to the several day delay to obtain pathological results. Due in large part

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to its recurrent nature and the shortcomings of cystoscopy for diagnosis and surveillance, bladder cancer is the most expensive cancer from diagnosis to death in the United States.1

Widespread interest exists in applying new technologies to improve the diagnostic accuracy of standard white light cystoscopy. The bladder is particularly well suited to evaluate novel endoscopic imaging modalities given the easy accessibility and the option of intravesical instillation of pharmacological agents to provide tissue contrast. Two promising technologies include hexylaminolevulinate fluorescence cystoscopy2 and optical coherence tomography.3 However, neither method provides the cellular resolution that could possibly reduce or eliminate the need for TURBT.

Confocal microscopy is a powerful imaging tool widely used in basic research.4 Using a laser light source and fluorescent dyes as contrast agents, confocal microscopy offers high resolution, dynamic, subsurface optical sectioning of biological systems.4–6 While conventional confocal microscopes are too large to use in vivo, recent advances in instrument miniaturization have led to the development of flexible, fiberoptic confocal microscopes that can be passed through the working channel of standard endoscopes.7–10 This enables in vivo microscopy, called confocal laser endomicroscopy or fibered confocal microscopy. Application of confocal laser endomicroscopy in the gastrointestinal tract for esophageal, stomach and colon cancer detection has moved beyond early proof of concept evaluation to large clinical trials.11–17 To date, in vivo use in the urinary tract has been limited to 2 animal studies.18,19

Recently we reported on confocal laser endomicroscopy of fresh radical cystectomy specimens using intravesical fluorescein, a Food and Drug Administration approved drug, as the fluorescent contrast agent.20 We noted cellular resolution and microarchitectural features of normal appearing and cancerous regions that correlated with standard hematoxylin and eosin staining.

In this study we hypothesized that confocal laser endomicroscopy could be used with conventional cystoscopy to provide real-time histological information that may help distinguish normal from neoplastic urothelium in vivo. We documented the confocal microscopic appearance of normal urothelium, and compared it to low and high grade tumors in patients undergoing TURBT.

MATERIALS AND METHODS

Confocal Microscopy Imaging System

The Cellvizio® fibered confocal microscope used in this study consisted of a 2.6 mm diameter flexible Confocal Miniprobe™ attached to a laser scanning unit. A 488 nm laser delivered the excitation beam for scanning that was transmitted through an imaging bundle containing more than 30,000 optical fibers. Several optical lenses at the distal end of the probe focused the beam into the tissue, producing images with a depth of penetration of 60 μm, a lateral resolution of 1 μm and a field of view 240 μm in diameter. Fluorescence was collected by the same lens and refocused back into the illumination fiber such that a single optical fiber acted as the illumination point source and the detection pinhole. The images were collected at a rate of 8 to 12 frames per second through direct contact of the urothelium with the probe tip. The prototype probes used for this study were sterilized with the STERIS® system before each use.

Imaging Protocol

The Stanford University and Veterans Affairs Palo Alto Health Care System institutional review board approved the research protocol (ClinicalTrials.gov Identifier NCT00801762). All patients scheduled to undergo TURBT based on abnormal office cystoscopy from September to December 2008 were recruited for the study. A single surgeon (JCL) performed each case including the confocal image acquisition. With the patient under spinal or general anesthesia standard white light cystoscopy was performed with a 21Fr rigid cystoscope or a 26Fr resectoscope with a visualizing obturator (Karl Storz Endoscopy, Culver City, California). Fluorescein sodium (International Medications Systems, Ltd., South El Monte, California) then served as a contrast agent through intravesical instillation and/or intravenous administration. For intravesical instillation 300 to 500 cc 0.1% fluorescein diluted in normal saline was instilled into the bladder via a Foley catheter and left indwelling for 5 minutes. For intravenous administration 1 ml 10% fluorescein was used. In subjects given fluorescein via both routes the intravesical fluorescein was always administered first. Endomicroscopy video sequences were obtained by passing the probe through the working channel of a standard Storz 26Fr resectoscope (fig. 1). This allowed for simultaneous confocal microscopy and white light cystoscopy using a 0-degree or 30-degree lens. Normal and abnormal appearing areas of urothelium were imaged. White light cystoscopy images and videos were recorded and stored on compact discs for each participant. Endomicroscopy video collection was controlled with a foot pedal, and the images were viewed in real time and stored as digital video files. In 9 cases biopsies were taken of the normal appearing area that had been imaged.

![Figure 1. A, 2.6 mm probe passed through working channel of 26Fr Storz resectoscope. Blue laser transmitted through fiber optic probe from Laser Scanning Unit. B, probe in direct contact with bladder urothelium during confocal imaging.](image-url)
with the confocal microscope to confirm the lack of malignancy. In another 11 cases in which intravenous fluorescein was used the prostatic urethra was also imaged.

After acquiring endomicroscopic images standard TURBT was performed using cold cup biopsy forceps followed by monopolar electrocauterization or the bipolar loop (Gyrus

Figure 2. Comparison of H & E and confocal endomicroscopic images of normal bladder mucosa with fluorescein staining. A, large, polygonal superficial cells consistent with umbrella cells. B, smaller, deeper urothelial cells consistent with intermediate cells. C, less cellular lamina propria containing blood vessels filled with erythrocytes.

Figure 3. A, endomicroscopic image of tumor resection bed after TURBT showing fibers of muscularis propria. B, H & E stain of biopsy from this site demonstrating fibers of muscularis propria. C, endomicroscopic image of resection bed revealing distinctive appearance of fat lobules. D, H & E stain of biopsy from this site confirming presence of fat in specimen.
ACMI, Southborough, Massachusetts). In 9 cases further endomicroscopic images were obtained after completing TURBT to evaluate the tumor bed. The biopsy specimens were then sent for standard pathological assessment with hematoxylin and eosin staining. The endomicroscopic images were further analyzed and compared with the hematoxylin and eosin sections of the corresponding tissues.

RESULTS
Between September and December 2008, 28 patients (mean age 73 years, range 47 to 90) were scheduled to undergo TURBT at our institution. All patients were recruited for the study and 1 declined, leaving a study population of 27 patients. Real-time endomicroscopy video sequences of the bladder mucosa were collected in vivo after intravesical and/or intravenous administration of fluorescein. Mean duration of the endomicroscopy portion was 18 minutes overall (range 3 to 35), 21 minutes for the first 13 cases and 16 minutes for the remaining 14 cases. No systemic toxicity or hypersensitivity reactions from intravesical or intravenous fluorescein were observed. Patients who received intravenous fluorescein had green tinged urine for approximately 24 hours.

Intravenous vs Intravesical Fluorescein
All patients received fluorescein intravenously (10), intravesically (5) or via both routes (12). Those given fluorescein via both routes underwent imaging after intravesical and again after intravenous fluorescein administration. The first subject received intravenous fluorescein before cystoscopy. However, due to the difficulty performing cystoscopy with the green fluorescein dye in the bladder, subsequent patients were given intravenous fluorescein only after completion of white light cystoscopy. We found subtle differences in image quality and contrast pattern between the 2 administration routes. Within 1 minute of injection of intravenous fluorescein, contrast material was visible in the blood vessels of the lamina propria, providing striking images of erythrocytes moving within the vessels. For several minutes the fluorescein diffused into the surrounding tissue providing contrast for visualization of bladder cellular architecture. After approximately 10 minutes the fluorescein began to be excreted into the bladder. With intravesical fluorescein image quality began to deteriorate after approximately 15 minutes, likely due to fluorescein washout. Interestingly in several patients given only intravesical fluorescein the vasculature in the lamina propria was visualized, suggesting that the fluorescein is able to diffuse through the transitional epithelium into the lamina propria. We were unable to image the bladder using the confocal probe without fluorescein due to the lack of significant autofluorescence of urothelial cells.

Normal Histology
Confocal laser endomicroscopy was performed on normal appearing areas of the bladder mucosa in all patients. We successfully visualized the normal bladder urothelium and lamina propria (fig. 2, video segment 1). Within the urothelial layer the more superficial cells were consistently larger than the deeper layer, corresponding to the known size difference between the larger umbrella cells and the smaller intermediate cells seen on hematoxylin and eosin microscopy. The urothelial cells existed in an organized network highlighted by the fluorescein staining of the extracellular matrix of the cells. This visualized pattern is unique to confocal microscopy because standard histopathological processing does not accentuate cellular borders and the tissue is cut in a plane transverse to the mucosal surface. Cellular nuclei were not visualized because fluorescein does not cross the cell membrane. However, cellular outlines were obvious due to the extracellular dispersion of fluorescein. By applying pressure with the probe the underlying lamina propria, with its vascular network and less cellular connective tissue, was visualized. Individual erythrocytes were consistently seen flowing within vessels of the lamina propria after giving intravenous fluorescein, and less commonly after intravesical administration. In general we noted decreased image quality with bladder overdistention. All 9 cases in which random bladder...
biopsies were obtained from a normal appearing area that had been imaged with the confocal microscope showed no evidence of malignancy. Endomicroscopic imaging of the resection bed after biopsy yielded images of muscularis propria fibers (fig. 3, A and B) and fat (fig. 3, C and D). In 2 of the 11 patients in whom the prostatic urethra was scanned images suggestive of prostatic glands and stroma were identified (fig. 4). In the other 9 patients blood vessels were visible but no glands or stroma were seen.

**Bladder Tumors**

Of the 27 patients 8 had no cancer (3 with normal urothelium only, 1 with cystitis cystica, 2 with inflammation only, and 1 with a dense lymphocytic infiltrate, and 1 with mild to moderate dysplasia and inflammation), 9 had low grade tumors, 9 had high grade tumors including 1 with carcinoma in situ, and 1 had a predominately low grade tumor with a high grade focus. Of the patients with high grade tumors 4 underwent subsequent radical cystoprostatectomy. In 2 patients endomicroscopic images were not obtained due to an anterior tumor location that made contacting the tumor with the probe tip impossible. Apart from these patients confocal laser endomicroscopy documented clear differences between normal mucosa, and low and high grade tumors in most cases. In contrast to the normal cellular architecture, low grade papillary tumors demonstrated densely arranged but uniformly shaped small cells extending outward from fibrovascular cores (fig. 5, video segment 2). High grade tumors showed markedly disorganized architecture and variably sized cells that were much different from normal urothelium or low grade tumors (fig. 6). Characterization of the nuclear appearance of the cells in these tumors was not possible because fluorescein does not enter the cells. In addition, with a 60 μm penetration depth the confocal probe used in this study cannot visualize the muscularis propria through the intact urothelium and, therefore, does not offer information about muscle invasion for staging.

**DISCUSSION**

We report the first in vivo microscopic evaluation of human bladder urothelium using a confocal laser endomicroscope. Confocal laser endomicroscopy enables subsurface imaging of living cells in the bladder mucosa during cystoscopy. Fluorescein, administered intravesically or intravenously, is a safe and effective contrast agent that permits visualization of the urinary tract with the confocal endomicroscope.
The confocal probe is passed through the working channel of a standard resectoscope, offering simultaneous confocal microscopy and cystoscopy. The acquired endomicroscopic images display sufficient resolution to distinguish cell size and architecture, allowing the differentiation of different depths in the urothelium and the lamina propria. Most importantly we noted marked differences between normal mucosa, and low and high grade tumors in most cases.

Our feasibility study has several limitations, particularly the relatively small sample size and the nonquantitative nature of the image analysis. In addition, due to the small size of the probe and the field of view, image acquisition is exquisitely sensitive to motion introduced by the subject (ie diaphragmatic motion or arterial pulsation) or the operator. Also, a probe developed for gastrointestinal endoscopy was used whose 2.6 mm size necessitates the use of a 26Fr rigid resectoscope instead of a flexible cystoscope. Lacking the ability to manipulate the probe tip with a rigid cystoscope makes establishing the required perpendicular contact with the tissue impossible in cases where the area of interest resides on the anterior bladder wall. Accordingly due to their location in the bladder some tumors in this study could not be imaged. In addition, given the 2.6 mm probe tip size with a 240 μm field of view and the requirement for direct contact with the urothelium, imaging the entire bladder is not practical. Rather the proposed role of confocal laser endomicroscopy is to provide real-time histology of areas highlighted by white light cystoscopy or other imaging modalities such as fluorescence cystoscopy.

Approximately 10 minutes after intravenous administration fluorescein is excreted into the bladder, potentially interfering with cystoscopic visualization. In contrast to most other hollow organs the bladder offers a direct route for topical administration of medications. Our study demonstrates that intravesical fluorescein instillation adequately provides cellular resolution and microarchitecture comparable to intravenous administration. In addition, intravesical fluorescein can be rinsed out of the bladder, thereby regaining excellent clarity while maintaining sufficient cellular contrast to permit microscopy. Nevertheless, image quality does deteriorate after approximately 15 minutes likely due to repeated bladder irrigation. However, intravesical fluorescein instillation may be repeated if necessary. Finally fluorescein does not permit visualization of cell nuclei as noted in similar studies in the gastrointestinal tract.15 Rather it nonspecifically stains the extracellular matrix of normal and abnormal bladder mucosa. Due to a concern of potential carci-

Figure 6. A, endomicroscopic image of high grade, noninvasive urothelial tumor with extensive architectural disarray and cellular irregularity. B, corresponding H & E slide. C, endomicroscopic image of another high grade tumor. D, corresponding H & E slide.
nogenicity we did not use acriflavine hydrochloride, a contrast agent capable of nuclear staining. Despite these limitations confocal laser endomicroscopy may prove to be a useful adjunct to conventional cystoscopy. The ability to differentiate normal, benign and neoplastic tissues in real time with microscopic resolution during cystoscopy would provide valuable diagnostic information that could impact clinical decision making such as avoiding unnecessary biopsies, allowing more targeted biopsies and confirming the low grade nature of tumors being managed expectantly. The possibility of using intravesical fluorescein to generate interpretable images makes the use of this technology in the clinic setting more feasible. To achieve that goal evaluation with smaller probes that can pass through a flexible cystoscope will be necessary. We are developing objective criteria for identifying and grading bladder tumors that will be applied in a larger prospective and blinded study in the future. A larger study would allow us to characterize the confocal microscopic appearance of benign bladder tumors to better distinguish them from bladder carcinomas. We also plan to image more patients with flat lesions such as carcinoma in situ, an application that may prove to be the best use of this technology.

CONCLUSIONS
Confocal microscopic imaging of the human bladder using fluorescein as the contrast agent can be performed in vivo and provides real-time imaging of the cellular architecture. In most cases marked differences were visualized among normal urothelium, low grade tumors and high grade tumors. Pending further clinical investigation and technological improvement, confocal laser endomicroscopy may become a useful adjunct to conventional cystoscopy.

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REFERENCES