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Comparison of Confocal Endomicroscopy and Immunohistochemical Localization of Fluorescein in Biopsy Specimens in Patients with Large Intestinal Neoplasms

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ABSTRACT

Background and objective : Confocal laser endomicroscopy (CLE) is a new diagnostic tool enabling virtual *in vivo* histologic examination of the mucosal layer during endoscopy, after intravenous injection of fluorescein. To understand the characteristic CLE images of neoplastic diseases, their findings were compared with immunohistochemical findings of biopsy specimens.

Patients and methods : The 31 lesions, including 6 hyperplastic polyps, 12 adenomatous polyps, and 13 colon carcinomas, were assessed. These lesions were observed with both routine colonoscopy and CLE. The CLE findings were compared with the results of the histopathological and immunohistological examinations of pinch biopsy specimens.

Results : On fluorescein immunohistochemical staining, both hyperplastic and adenomatous polyps were the same as normal colon tissue ; staining was localized in the interstitium and at the mucosal surface of the crypts. The enterocyte cytoplasm, but not the nuclei, was stained. These staining patterns were consistent with the localization patterns seen on CLE images. The stain distribution noted in the colon cancers was similar to that seen in the polyps, but both the pattern and intensity of staining were irregular. These immunohistochemical findings were consistent with the fluorescein localization patterns seen on CLE images.

Conclusions : CLE suggests very useful for *in vivo* assessment of colonic lesions.

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Key words : confocal laser endomicroscopy, fluorescein, colonic neoplasm

INTRODUCTION

Diseases of the large intestine have been diagnosed with increasing frequency and include neoplasms, inflammatory disorders, and psychosomatic disorders. The proper diagnosis of these diseases guides further treatment, and colonoscopic examination is considered the gold standard for diagnosis[1-3].

In addition, high-resolution video endoscopy and numerous technological advances, such as chromoendoscopy[4], magnifying endoscopy[5-7], narrow-band imaging (NBI) endoscopy[8], optical coherence tomography (OCT)[9,10], and endoscopic ultrasonography (EUS), provide a wealth of information that allows accurate diagnosis and further treatment[11]. However, in almost all cases, the pinch biopsy obtained during colonoscopic examination is considered the gold standard for diagnosis, despite the risks associated with biopsies, such as bleeding and perforation. Even in patients receiving anticoagulant therapy, a pinch biopsy may be required to make a definitive diagnosis. However, biopsies are associated with

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sample error, because the pinch biopsy may not permit evaluation of the entire lesion, and are costly. Thus, the development of a virtual biopsy method that enables a histopathological diagnosis without pinch biopsy has been pursued[12].

Confocal laser microendoscopy (CLE) (OptiScan Ltd., Victoria, Australia, and HOYA Ltd., Tokyo, Japan) is newly developed method of colonoscopy[13,14]. CLE is an approach that has shown much promise for virtual biopsy[13–15]. The CLE that was used is based on the integration of a confocal laser system in the distal tip of a conventional video colonoscope. The technique is an adaptation of standard light microscopy in which low-power laser illumination is focused to a single point in the microscopic field of view (FOV). The fluorescent images have a high magnification (up to 1,000 times), a high resolution (0.5–1 μm), a focal imaging depth of 250 μm, and a field of 500 μm^2.

The CLE images consist of the fluorescein localization signals. Thus, it is important to assess the localization of fluorescein immunohistochemically. We have previously reported on the localization of fluorescein in normal tissue from the large intestine and found that CLE may be a suitable tool for performing virtual biopsies[16]. In the present study, CLE images of neoplastic diseases, such as hyperplastic polyps, adenomatous polyps, and carcinomas, were compared with the localization of fluorescein using immunohistochemical techniques in biopsy specimens.

PATIENTS AND METHODS

All patients undergoing CLE gave their written informed consent, and the protocol was approved by the Research Ethics Committee of The Jikei University School of Medicine. The study cohort consisted of 51 patients (35 men and 16 women) whose average age was 64 years (range, 38 to 77 years) and who were seen from April 2004 through October 2006. The colonoscopic examinations were performed because of occult-blood-positive stool results, follow-up after polypectomy and colonoscopic mucosal resection (EMR), and preoperative marking. Six cases of hyperplastic polyps, 12 cases of adenomatous polyps, and 13 cases of colon cancer were included.

Pretreatment used for CLE was the same as that used for routine colonoscopy. The colon detergent Niflec® (Ajinomoto-Pharma Ltd., Tokyo, Japan) was used for colonoscopic lavage. Just before the examination, the patient was given 2 mg of intravenous midazolam for sedation, as well as 5 mg of scopolamine butylbromide and 1 mg glucagon to decrease intestinal spasm. During colonoscopy, a pulse oximeter was used to monitor arterial oxygen concentration, and an electrocardiographic monitor was used to monitor the cardiac status.

Once the colonoscope had reached the cecum, the routine colonoscopic images were examined, and the CLE images were assessed immediately after fluorescein had been given intravenously. The dose of fluorescein sodium was 10 mg/body weight kg, to a total dose of 500 mg.

When a lesion was found, the routine colonoscopic images and CLE images were observed, and pinch biopsies were obtained. The biopsy specimens were stained with hematoxylin and eosin for histopathological diagnosis, and the localization of fluorescein was assessed with immunohistochemical methods.

For the immunohistochemical examinations, the avidin-biotin complex (ABC) immunoperoxidase technique (Vectastain ABC Kit ; Vector Laboratories Inc., Burlingame, CA, USA) was used. Mouse monoclonal antibody to fluorescein (Abcam Ltd., Cambridge, UK) was used as the primary antibody. Formalin-fixed, paraffin-embedded thin sections were deparaffinized with a graded series of ethanol and xylene, and hydrogen peroxide (3%) in distilled water was applied for 10 minutes to block endogenous peroxidase. After being washed in phosphate-buffered saline (PBS, pH 7.4), the sections were covered with 1.5% normal goat serum in PBS for 20 minutes, and then incubated with primary antibodies in a 1 : 2,000 dilution overnight at 4°C. After being washed in PBS, the sections were incubated with a biotinylated secondary antibody for 30 minutes. This incubation was followed by incubation with avidin-biotin peroxidase complex reagent for 30 minutes and then washing in PBS. The bound complexes were visualized with diaminobenzidine

(Sigma-Aldrich, St. Louis, MO, USA) in Tris buffer containing 0.067% hydrogen peroxidase. The sections were counterstained with hematoxylin and then mounted.

Normal, surgically resected colon tissue was used as a negative and positive control. The normal tissue adjacent to lesion in surgically resected colon was used as the positive control. Normal colon into which a diluted fluorescein solution was directly injected was compared by means of immunohistochemical methods with tissue into which fluorescein was not injected on the same slides.

Results

No patients in this study had adverse effects or complications from either the injected fluorescein or the CLE procedure. When fluorescein was injected intravenously, the surface of the colon became yellowish; this change in color did not pose any problems for the routine colonoscopic observations. The CLE images appeared an average of about 15 seconds after the intravenous injection of fluorescein. After the CLE images had become visible, they were obtained for 20 minutes without any difficulties. Thus, routine colonoscopic images and CLE images of the large intestine were obtained over a period of 20 minutes after fluorescein injection.

1. *Hyperplastic polyps*

The CLE images were consistent with the histopathologic findings of biopsy specimens (Fig. 1a). On CLE, stellate crypts without atypia were observed.

Positive signals were strong in the interstitium, weak in the cytoplasm of enterocytes, and not present in enterocyte nuclei or in goblet cells (Fig. 1b). The CLE images of the hyperplastic polyps were consistent with the histological findings and immunohistochemical fluorescein localization noted in the pinch biopsy specimens; however, these findings were not characteristic of hyperplastic polyps.

2. *Adenomatous polyps*

The CLE images were consistent with the histopathologic findings of biopsy specimens (Fig. 2a). On CLE images, tubular crypts of various size without atypia were observed.

Although tubular crypts of various size and shape were observed, the locations of positive signals were similar to those seen in normal colon tissue. The positive signals were strong in the interstitium and the cytoplasm of enterocytes but were not present in enterocyte nuclei or goblet cells (Fig. 2b).

The CLE images of adenomas were consistent with the histological findings and the immunohistochemical fluorescein localization seen in the pinched biopsy specimens. The characteristic findings of the histological structures were seen on the CLE images, but these findings were not characteristic of adenoma.

3. *Colon carcinomas*

The CLE images were consistent with the histopathologic findings of biopsy specimens (Fig. 3a). On CLE images, the crypts had typical cellular and structural atypia, which was consistent with the histopathological findings of the biopsy specimens. Although the CLE showed strong signals throughout carcinoma lesions, the signals were irregular. In every lesion, both the enterocytes and the interstitium showed strong fluorescein signals, but the nuclei showed no signals. On immunohistochemical examination, the fluorescein localization was consistent with that seen on the CLE images. Fluorescein was observed in all enterocytes of the entire crypt and the interstitium; within the enterocytes, fluorescein was seen in the cytoplasm but not in the nuclei (Fig. 3b).

Discussion

CLE is a revolutionary tool that provides clear, real-time microscopic images at a histopathological level during colonoscopy. CLE is useful for diagnosing colorectal cancer *in vitro*[13] and for detecting colorectal dysplasia/carcinoma during follow-up of patients with ulcerative colitis[17].

In our previous reports[16], the localization of fluorescein in normal large intestinal tissue was found to correspond well with the actual CLE images. The CLE images were seen after the fluorescein had been administered. The intravenously administered fluor-

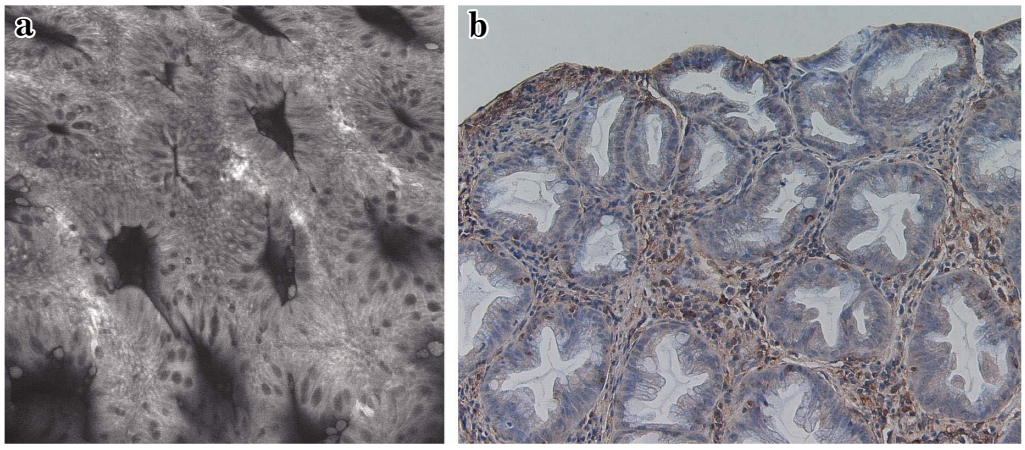

Fig. 1. a. CLE image of a hyperplastic polyp
The CLE image shows a stellate tubular structure that is common in hyperplastic polyps; no atypical structures are seen. Fluorescein signals are observed in the interstitium and cytoplasm but not in the nuclei of enterocytes in the crypt (FOV = 500 μm).
b. IHC at low magnification
There was significant overlap between the fluorescein seen in the CLE image and on immunohistochemical studies: in both, the fluorescein was located in the interstitium and cytoplasm but not in the nuclei of enterocytes in the crypts that are characteristic of hyperplastic polyps.

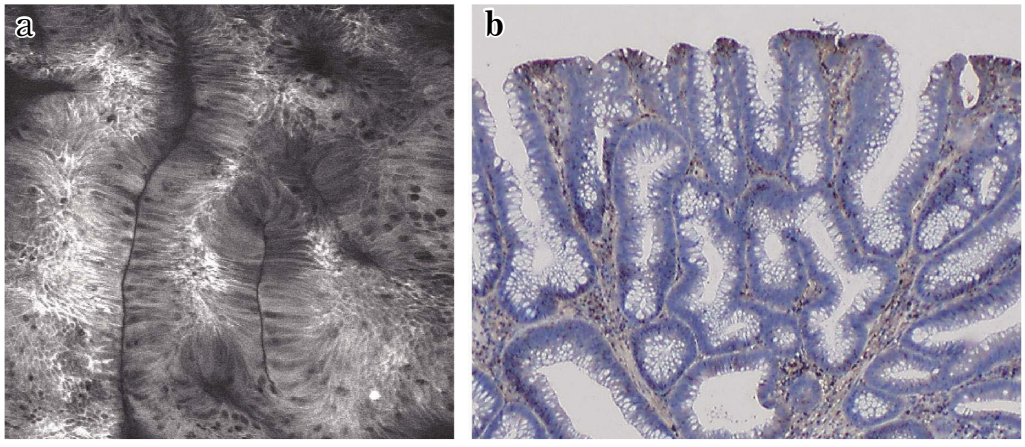

Fig. 2. a. CLE image of an adenomatous polyp
The CLE image shows the tubular structure that is characteristic of adenomatous polyps. Fluorescein signals are observed in the interstitium and cytoplasm but not in the nuclei of enterocytes within the tubular structure. Of note, the fluorescein signal is weaker in the cytoplasm than in the interstitium (FOV = 500 μm).
b. Immunohistochemical staining at low magnification
The fluorescein signals correspond with those seen on CLE. In both, the fluorescein signals are located in the interstitium and in the cytoplasm but not in the nuclei of enterocytes within the tubular structure of the adenomatous polyp.

escein first appears at the capillary loop that surrounds the crypts and then spreads towards the interstitial tissue. Finally, the fluorescein shows the enterocytes lining the crypts and is then released into the intestinal lumen. Curiously, goblet cells do not show fluorescein signals. The actual CLE images are similar to those obtained with immunohistochemical techniques. Important information can be obtained from the microscopic CLE images obtained during colonoscopy. The CLE images differ during the various phases following fluorescein administration. Thus, the CLE images can be used not only for ultrastructural observation but also to evaluate function.

In practice, it is important to be cognizant of the fluorescein localization patterns in typical diseases, such as hyperplastic polyps, adenomatous polyps, and

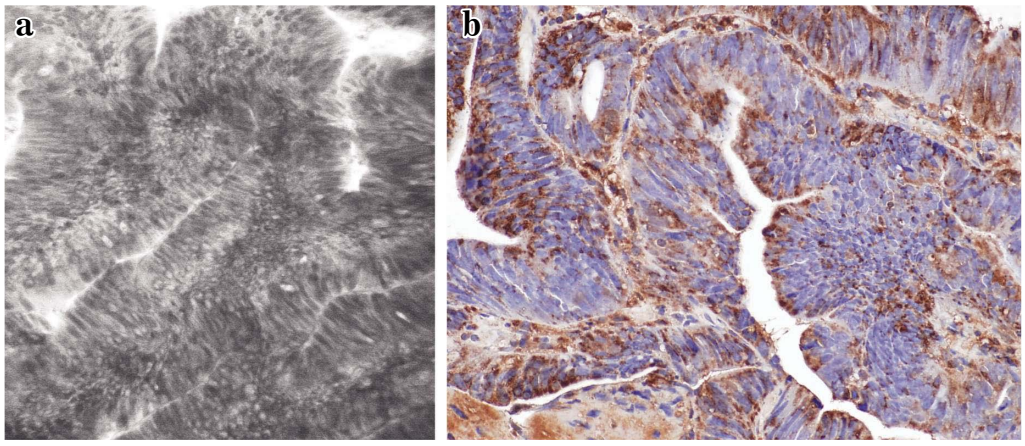

Fig. 3. a. CLE image of an adenocarcinoma
The CLE image shows the structural and cellular irregularity that is characteristic of adenocarcinoma. Fluorescein signals are observed in the interstitium and in the cytoplasm but not in the nuclei of the adenocarcinoma lesion (FOV＝500 μm).
b. Immunohistochemical staining at low magnification
The fluorescein signal localization corresponds with that seen on CLE. Although the intensity of the fluorescein signals varied and differed between hyperplastic and adenomatous polyps, the localization of these signals was similar to in hyperplastic and adenomatous polyps; staining can be seen in the interstitium and in the cytoplasm but not in the nuclei of enterocytes of the adenocarcinoma.

carcinomas. Therefore, CLE images and immunohistochemical results for the 3 typical lesions were compared.

Fluorescein and acriflavine are commonly used as contrast agents for CLE because of their convenience and low incidence of adverse effects. The fluorescein used in the present study can be given either intravenously or using a direct spray method. In our experience, intravenous fluorescein injection provides better CLE images of each layer. As previously reported[16], strong fluorescein signals are localized in the interstitial tissue and in the cytoplasm of enterocytes within the crypts but not in goblets cells or enterocyte nuclei.

In both hyperplastic and adenomatous polyps, the localization of the fluorescein signals was the same as in normal colon tissue. On immunohistochemical examination, the localizations of the fluorescein signals were found to be in the interstitium and the cytoplasm of enterocytes but not in the nuclei of enterocytes or in goblet cells. There was no major difference between hyperplastic and adenomatous polyps in the localization of fluorescein signals, but there were structural differences. Hyperplastic polyps showed stellate crypts of similar size, and the adenomatous polyps showed tubular structures ; these findings corresponded to the histological characteristics observed in the specimens obtained at biopsy or surgery.

The findings in colon carcinoma were similar to those in the hyperplastic and adenomatous polyps. The immunohistochemical location of fluorescein signals was found in the interstitium and in the cytoplasm of enterocytes but not in the nuclei of enterocytes or goblet cells, which were very few. However, the intensity of the fluorescein signals differed even within the same specimen. Furthermore, the structure of carcinoma lesions differed from that of normal colon. On CLE, the carcinoma lesions showed large structural and cellular irregularities, similar to those seen on histological examination, and colon carcinoma lesions showed the structural and cellular characteristics of carcinoma, with various degrees of intensity of the fluorescein signal in struc tures that had a positive signal. These findings might complicate diagnosis, even though they are consistent with carcinoma. The degree of disappearance of normal function might depend on the differentiation of the carcinoma ; the results of the present study might be related to the degree of disappearance of normal

function in a particular case of colon carcinoma.

Typical carcinoma could be diagnosed on the basis of routine colonoscopy and CLE images. For lesions in which it is hard to distinguish between carcinoma and adenoma with a high degree of atypia, a definitive diagnosis is difficult to establish, even with biopsy and surgical specimens. Clinically, it is very important to distinguish carcinomas from adenomas, because metastasis to regional lymph nodes must be considered in cases of carcinoma. The same difficulty in diagnosis may occur with CLE. In the present study, typical histological features of colon carcinoma were observed which were consistent with the pathological findings, which is useful information for making a definitive diagnosis.

However, in complicated lesions, such as adenoma with high-grade atypia and well-differentiated adenocarcinoma, both structural atypia and cellular atypia must be assessed, as is done during histopathological examination. In such lesions, simultaneous use of another contrast agent, such as acriflavine, which can show the cytoplasm and the nucleus following direct spray application, may provide crucial information on CLE.

CONCLUSIONS

These observations provide further confirmation that CLE is useful for *in vivo* assessment of colonic lesions.

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