四国歯誌　21（2）：333～339，2009

上村修三郎「がん研究」奨励賞受賞講演

Molecular Imaging in Endoscopic Oncology

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Keywords：infrared fluorescence, probe, gastrointestinal cancer

Abstract：The goal of our study is to develop a molecular imaging in gastrointestinal tract using infrared fluorescence endoscope (IRFE) and labeled antibodies with indocyanine green (ICG)-derivative in detecting cancerous tissue. IRFE consisted of an infrared endoscope equipped with excitation (710-790 nm) and barrier (810-920 nm) filters. We have developed ICG-N-hydroxy sulfo succinimide ester (ICG-sulfo-OSu) and 3-ICG-acyl-1,3-thiazolidine-2-thione (ICG-ATT) as an infrared fluorescent-labeling reagent. ICG-derivative-labeled anti-human carcinoembryonic antigen (CEA) antibody and MUC1 antibody were prepared. Freshly resected specimens with gastric cancer were observed by IRFE after reaction with ICG-derivative labeled antibodies. It resulted in positive fluorescence at the tumor location by IRFE, and the immnofluorescent images correlated well with the tumor sites. The immunohistochemical studies suggested the intensity of IR fluorescence of ICG-ATT-MUC1 stronger than ICG-sulfo-OSu. We conclude that an anti-CEA or MUC1 antibody with affinity for cancerous lesions and labeled with ICG-derivative can be imaged with this IRFE. Specific antibodies labeled with ICG-derivative with the reinforcing agent and electronic device can visualize cancer proteins generating a strong enough fluorescent signal.

1. Introduction

Diagnostic endoscopy has been used for early detection of gastrointestinal neoplasias[1)]. Diagnostic accuracy can be enhanced by good training and adequate experience, but diagnosis using conventional endoscopy is essentially limited because it is based on morphological changes and/or discoloration[2, 3)]. This limitation is overcome by histopathological diagnosis of a biopsy specimen, but biopsy takes extra time and expense and is not advisable for patients taking anti-coagulants or anti-platelet medicine. Thus, the future of endoscopic diagnosis is likely to be a combination of biomarkers and instrumental technology. ‘Molecular imaging’ is a concept representing the most novel imaging methods in the field of endoscopy, which have been called ‘immunoscopy’[4)], ‘bioendoscopy’[5)], and ‘optical biopsy’[6)]. We have attempted to develop a novel imaging method using antibodies labeled with a fluorescent marker and imaging modalities[7)].

2. Characteristics of infrared fluorescence

Infrared radiation is light with wavelengths between 780 nm and 100 μm, and it has high permeability and safety compared to ultraviolet rays. These characteristics have been applied to various technologies such as non-destructive analysis of agricultural products, and infrared photography has been especially investigated in the medical field[8, 9)]. Infrared endoscopy has been used as a special diagnostic tool for examination of the gastrointestinal tract with or without intravenous injection of indocyanine green (ICG)[10-13)]; ICG is widely used as a reagent for clinical examination of hepatic function[14)]. ICG is a fluorescent agent that absorbs infrared rays and produces visible spots at the maximum

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wavelength of 805 nm[15]. ICG emits wavelengths of 807-832 nm on excitation at around 770 nm[16-18]. Taking advantage of this characteristic, infrared fluorescence is used for retinal angiography[19] and the evaluation of burn depth[20] and the patency of cardiac venografts[21]. Recently, this property was applied to gastrointestinal blood vessels using a CCD camera[22]. In the living body, components or elements emit fluorescence of 310-540 nm when excited at 280-370 nm. In addition, there is little background noise in the living body[23], especially in the digestive tract, which makes infrared fluorescence a likely candidate for development as a novel diagnostic system[24, 25]. Several kinds of labeling agents for detecting carcinomas in the digestive tract have been reported[26-28], some of which fluoresce in visible or ultraviolet rays. However, application of ultravidet is not suitable because it damages living tissue[29]. ICG seems to be a suitable molecule for immunofluorescent diagnosis in the digestive tract such as esophagus, stomach, and colon because of its spectral properties and low toxicity.

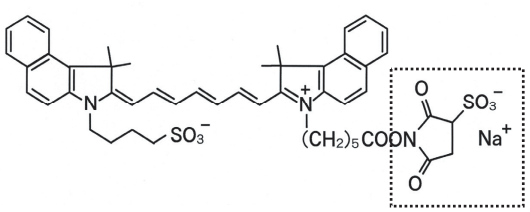


Fig. 1 The chemical structure of ICG-sulfo-OSu. ICG-sulfo-OSu has an ester group, a characteristic chemical structure capable of binding to various antibodies (squared).

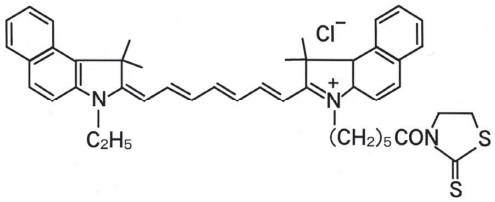


Fig. 2 The chemical structure of ICG-ATT. ICG-ATT is also an infrared fluorescent-labeling reagent useful for proteins and amino acid compounds.

3. Fluorescent agents

Although ICG binds albumin in a non-covalent way in the blood, it lacks a protein-binding group to bind antibodies. Therefore, we developed an ICG-N-hydroxysulfosuccinimide ester (ICG-sulfo-OSu) (Fig. 1) that has the ability to bind to proteins[30]. The physiochemical characteristics resembled those of ICG: the absorption maximum was 795 nm, and it has a specific fluorescence emission at 807 nm upon excitation at 768 nm. However, the fluorescence intensity was not sufficient when it was labeled with an antibody. Consequently, Nagao et al. developed ICG-acylthiazolidinethione (ICG-ATT) (Fig. 2)[31], which consists of the ICG skeleton, an alkyl side chain, and the thiazolidinethione amide group. The absorption maximum was 789 nm, and the fluorescent maximum was 830 nm upon excitation at 765 nm, reflecting the structure of the original ICG-dye moiety. Both materials proved to be near-infrared fluorescent agents, but it is unknown if ICG derivatives are toxic to living body in a clinical setting. Although precise toxicity tests have not been performed yet, ICG derivatives are expected to be non-toxic because the basic structures of these materials are similar to ICG, which is non-toxic.

4. Labeled antibodies

First, we labeled an anti-epithelial membrane antigen (EMA) antibody with ICG-sulfo-OSu. Although the anti-EMA antibody is not cancer-specific, this antibody is cross-reactive with normal epithelium in the digestive tract and has a relatively high sensitivity[32]. Therefore, we first confirmed the sensitivity and specificity of immunofluorescence of the labeled antibody. Anti-EMA antibody (Dako, Denmark) was labeled with ICG-sulfo-OSu by our standardized method. Anti-EMA antibody (2.8 mg) was dissolved in 4 ml of 100 mM sodium bicarbonate buffer (pH 8.5), and 40 μl of 6 mM ICG-sulfo-OSu dissolved in dimethylsulfoxide was added, followed by incubation at 37℃ for 1 hour. Based on our previous study, the molecular ratio of anti-EMA antibody to ICG-sulfo-OSu was 1:12. The reaction mixture was purified with a Sephadex G-25M column (PD-10, Pharmacia, Sweden) using 50 mM phosphate-buffered saline (PBS) as an eluent. The dye-conjugated antibody was separated from the free dye. The greenish solution of ICG-sulfo-OSu labeled anti-EMA antibody was immediately freeze-dried. The physiochemical characters of the labeled antibody were similar to those of ICG. After confirmation of adequate immunofluorescence from this labeled antibody, we attempted to develop a cancer-specific labeled antibody. In fact, various cancer-specific antibodies, such as the anti-carcinoembiyonic antigen (CEA) antibody, have been used as labeled antibodies for the diagnosis of gastrointestinal cancer[33]. We have also labeled an anti-CEA antibody (Chemicon International Inc., CA, USA), which has a high sensitivity for gastrointestinal cancer[34], with ICG-sulfo-OSu. The excitation and emission spectra of ICG-sulfo-OSu labeled anti-CEA antibody was also similar to that of ICG[18]. Although the labeled anti-CEA antibody showed efficient immunofluorescence, we developed a new labeled antibody using a more sensitive tumor marker. Mucin, a glycoprotein containing a large amount of sugar, is the main

component of mucus, and the peptide structure of the mucin core protein has been clarified[35]. The specific expression of mucin in various cancers has been reported, and we also studied the stainingpattern and evaluated its sensitivity in gastrointestinal cancers [36]. Based on its relatively high sensitivity[37, 38], we labeled an anti-MUC1 antibody (MY.1E12; kindly provided by Prof. Tatsuro Irimura, The University of Tokyo) with ICG-ATT.

5. Imaging modality

Based on the characteristics of ICG-derivatives, a prototype of infrared fluorescence endoscopy was developed to observe the human gastrointestinal tract (Fig. 3)[29]. The system consisted of an infrared endoscope (Olympus XGIF-Q40IR, Olympus) coupled with an image-capturing device. The light source, a 300 W xenon lamp, was also equipped with an excitation filter and a barrier filter, making it was possible to observe fluorescence with the infrared excitation light and produce normal images under visible light. The Intensified CCD (ICCD) camera was optically connected with the scope through an adapter into which the barrier filter was inserted. The new endoscopy system with a CCD at its tip has a greatly improved resolution: this system comprises of a light source apparatus, an infrared fluorescence endoscope, and image analysis software, which is the same as that used for a conventional system[40]. The light source apparatus (XCLV-260HP-IRF, Olympus) has three built-in filters; an infrared ray cut filter, an infrared ray pass filter, and a RGB filter. White light produced by a xenon arc lamp goes through the infrared ray cut filter or infrared ray pass filter and then through the RGB rotation filter. The infrared ray cut filter is used for conventional observation and the infrared ray pass filter for infrared fluorescence observation. The infrared ray pass filter can transmit rays of wavelengths between 540 nm and 560 nm in addition to infrared rays of wavelengths between 680 nm and 770 nm. The reflected light of the former rays allow us to know where in the stomach we are looking during infrared fluorescence observation. The RGB rotation filter can transmit light in the near-infrared region as well as the visible region. The infrared fluorescence endoscope (XGIFQ-240IRFZ, Olympus) is equipped with both a CCD with high resolution for conventional observation and a CCD with high sensitivity for infrared fluorescence observation at the top. These two CCDs can be switched from one to another with a single touch of the button of the endoscope in conjunction with the switch from one filter to another (the infrared ray cut filter and infrared ray pass filter) in the light source apparatus. Infrared rays excite the ICG-derivative-labeled antibody to emit fluorescence, which is subjected to barrier filters (825 to 945 nm) placed in front of the CCD for infrared fluorescence and is monitored as green signals on a pseudo-color display with an image processor (XCV-260HP-IRF, Olympus).

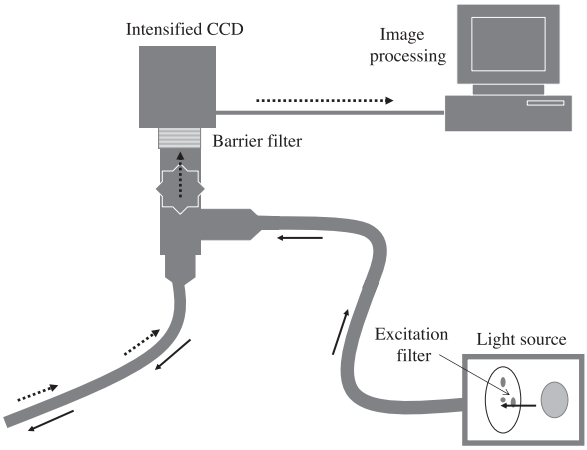


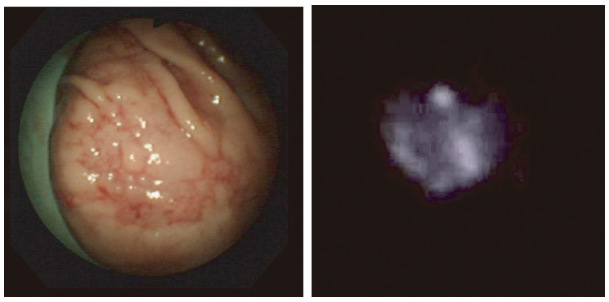
Fig. 3 The schema of infrared fluorescence endoscope system. This system consisted of an infrared endoscope (Olympus XGIF-Q40IR, OLYMPUS) coupled with an image-capturing device. The light source that includes a 300 W xenon lamp which was equipped with an excitation filter and a barrier filter, so that it was possible to observe fluorescence with infrared excitation light and normal images under visible light. The ICCD camera was optically connected with the scope through an adapter into which the barrier filter was inserted (Ito S, et al. Endoscopy 33: 849-853, 2001).

6. *Ex vivo* study of immunofluorescence with human stomach

The resected stomach tissue was treated with warm water of 37℃ containing 20000 U of Pronase, 1 g of $NaHCO_3$, and 4 mg of dimethylpolysiloxane for 15 min at room temperature to remove mucus adhering to the mucosa. The sample was treated with normal horse serum (blocking serum) for 15 min. The surface of the lesion and normal mucosa were then treated with ICG-derivative-labeled antibody for 60 min at room temperature[39]. Then, normal mucosa and the cancerous area were compared using the infrared fluorescence endoscope. The immunofluorescence of CEA labeled with ICG-sulfo-Osu was strongly recognized, while there was no obvious fluorescence in the normal mucosa (Fig. 4a, b). Paraffin sections of the specimen were stained with CEA antibody, and the fluorescence-positive sites and the immunoreaction with non-labeled CEA antibody corresponded well.

7. Reinforcement of fluorescence intensity

Some types of drugs (octylglucoside, OG) are known to be useful as reinforcement agents[41]. The peak fluorescence



a b

Fig.4 Infrared fluorescence imaging of freshly resected human gastric cancer tissue using ICG-sulfo-OSu-labeled anti-human carcinoembryonic antigen (CEA) antibody and an infrared fluorescence endoscope. The cancerous areas were stained with the fluorescent anti-CEA antibody complex. **a**. The image observed under visible rays. **b**. The image observed under infrared rays (Ito S, et al. Endoscopy 33: 849-853, 2001).

wavelength lengthened with increasing concentration; an increase in the OG concentration from 10 to 100 mM resulted in a shift of the peak fluorescence wavelength from 800 to 817 nm. In a study with paraffin sections of human gastric cancer, slight fluorescence was observed without OG; however, with 100 mM OG, marked fluorescence was observed[42]. In another study, we assessed the relationship between the fluorescence and protein ratio (F/P ratio) and fluorescence intensity[43]. During purification of the labeled antibody, the concentration of each labeling compound reacting with 1 molecule of the antibody was varied as follows: 4, 8, 16, and 32 molar equivalents. Subsequently, the intensity of fluorescence was evaluated by spectroscopy and infrared fluorescence microscopy. When the fluorescent antibody labeled with ICG-ATT was used at an F/P ratio of 2.94 or 4.18, clear and specific fluorescent images of the antigen were obtained. When the ICG-ATT-labeled antibody was used at an F/P ratio of 6.50 or 6.75, the fluorescence intensity decreased and the fluorescent images of the antigen became unclear. Therefore, the lower binding molar ratios of ICG-ATT were more useful for labeling the antibody. In previous studies, the whole IgG molecule was commonly used for preparation of labeled antibodies. However, labeled IgG displays insufficient sensitivity and specificity, probably resulting from nonspecific binding of the Fc fragment to target cells or molecular structure-dependent interference between fluorochromes. We characterized an Fc-free fluorescence-labeled Fab fragment, which was expected to yield more-specific binding to target cells than the whole IgG molecule. An anti-mucin antibody and ICG-ATT were used as the labeled antibody and labeling compound, respectively. Paraffin sections of excised gastric cancer tissues were subjected to staining. The labeled whole IgG molecule (ICG-ATT-labeled IgG) and the labeled Fab fragment (ICG-ATT-labeled Fab) were prepared according to a previous report, and the features of fluorescence microscopy images obtained from paraffin sections were compared. The fluorescence intensity obtained from paraffin sections of excised gastric cancer tissues tended to be greater with ICG-ATT-labeled Fab than with ICG-ATT-labeled IgG. Fragmentation of antibodies is considered to contribute to improved sensitivity and specificity of labeled antibodies for detection of micro gastrointestinal cancers[44].

8. *In vivo* reaction for exogenous antibody

In vivo immunostaining is essential for utilization of this technique in endoscopic diagnosis; however, no method has been established yet[45]. We examined in vivo immunostaining using nude mice. A human gastric cancer was transplanted into the mice, and the tumor was exposed under ether anesthesia. A tissue sample was collected after treatment with an antibody, and immunostaining was performed using the avidin-biotin complex method. Where anti-MUC1 mucin antibody had been applied in vivo to the cleaved surface of the grafted gastric cancer, the reaction product was demonstrated by the luminosity of the neoplastic tissue. In positive controls, strong reactivity was seen, but in the negative control, reactivity was not observed[46].

9. Future perspectives in diagnostic endoscopy

Diagnostic accuracy in endoscopic imaging can be enhanced by better training, including education and experience in lesion recognition and morphological diagnosis. An important characteristic of electroendoscopy is the ability to isolate lesions to facilitate their detection and qualitative diagnosis. There are numerous methods for image enhancement and adjunctive techniques that rely on the differential tissue effects of lights[2, 47-53]. Techniques exploiting previously unused properties of light have demonstrated the potential to enhance the ability to make clinical diagnoses without removing tissue as has been standard practice of decades. "Optical biopsy" is term that describes an image enhancement system that can detect dysplasia and neoplasia in real time. Although this technique is not yet widely available, enthusiasm for such techniques has grown, as has research in their potential clinical utility[54]. In the future, diagnosis will be based on the fact that diseases are defined by their molecular characteristics, rather than their morphology. "Endoscopic molecular imaging" to visualize the enzymatic and protein properties of tissues will be possible. When this procedure is realized, biomarkers will take a more important role in imaging.

However, at present, manufacturing limits future technologic

innovation in gastroenterology. Pharmaceutical companies are hesitant to invest in the development of novel diagnostic drugs, owing to the inability to precise predict marketing in this field and anticipate reimbursement, leading to loss of opportunities for the acquisition of advanced technology[3, 24]. It is important that instrumentation and drugs be developed and promoted efficiently through the cooperation of the industry, national grants, and the efforts of the academic sectors[55].

This work was supported in part by Uemura Shusaburo Foundation.

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