REVIEW

Principle and clinical usefulness of the infrared fluorescence endoscopy

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Abstract : Since there is no infrared fluorescence materials in the living body, infrared fluorescence labeling materials are very useful for making a diagnosis of a micro cancer. We have developed an infrared fluorescence endoscope (IRFE) and indocyanin green (ICG)-derivative as infrared fluorescence labeling materials to evaluate gastrointestinal neoplastic lesions. The study aims were to apply an IRFE and to demonstrate its usefulness in detecting cancerous tissue using an antibody coupled with ICG-derivative.

IRFE consisted of an infrared endoscope equipped with excitation (710-790nm) and barrier (810-920nm) filters and an intensified CCD camera. 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 mouse anti-human carcinoembryonic antigen (CEA) antibody and MUC1 antibody were employed in this study. Moreover, we examined the ability of a reinforcement agent, octylglucoside, to intensity fluorescence from the labeled antibody. Biopsy specimens of gastric cancer were stained with anti-CEA antibody by the avidin-biotinylated peroxidase complex method. Among the positive specimens, freshly resected stomach from three cases were used for the infrared (IR) imaging analysis.

The incubation of freshly resected stomach specimens with ICG-anti-CEA antibody-complex resulted in positive staining of the tumor sites by IRFE, and the IR fluorescent images correlated well with the tumor sites. The immunohistochemical studies suggested that the intensity of IR fluorescence of ICG-ATT-MUC1 was stronger than that of ICG-sulfo-OSu. In tumor sections, the reinforcement agent intensified fluorescence, ever at low antibody concentrations.

Therefore, we conclude that an anti-CEA (and/or MUC1) antibody with affinity for cancerous lesions and labeled with ICG-derivative can be imaged with this IRFE.

Specific antibodies tagged with ICG-derivative with the reinforcement agent can label cancer cells and generate a strong enough fluorescent signal to detect small cancers when examined with an IR fluorescence endoscope. J. Med. Invest. 53 : 1-8, February, 2006

Keywords **:** *infrared fluorescence, infrared fluorescence endoscopy, indocyanin green delivative, endoscopic diagnosis, bioendscopy*

INTRODUCTION

We had been studied about gastrointestinal endoscopy for a long time. However, sometimes, it is very difficult to make a diagnosis for small cancerous lesions. In order to make a diagnosis for small cancerous lesion, we should keep special endoscopic technique. Although

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we made all efforts that we could, it was very difficult for us to make a diagnosis of microcancer. Thus, changing the way of thinking to that of an ordinary person, we have established a new type of diagnostic method that requires no particular expertise.

The progress of medical instruments is remarkable. Recently, fiberscopes have increasingly been replaced by videoendoscopes. Videoendoscope is characterized by live pictures as seen on television. Because images are processed as electronic signals in videoendoscope, diagnosis of microcancer, which is usually undetectable through endoscopy, can be made in examination of a micro lesion that is invisible through macroscopy if the lesion is labeled with an antibody to be identified by videoendoscope and electronic signals from the labeled substance are processed by a computer to produce images. However, new diagnostic system using special characteristic of videoendoscope has not been established.

To establish this method, the following steps were required:1) the development of a biologically applicable labeled substance, 2) the development of a videoendoscope that is able to detect the labeled substance, and 3) the establishment of a vital immunohistochemical staining.

Fluorescence-labeled substances are good candidates for step #1. However, most known fluorescence-labeled substances fluoresce in the presence of ultraviolet (UV) irradiation. Moreover, there are many biological tissues that can spontaneously fluoresce when irradiated with UV and visible light, causing background noise.

We had a vague idea that it might become possible to establish a new diagnostic system. At the time when we thought such an idea, the Gulf War broke out. At the US-Iraq talks, James Baker, the former Secretary of the U.S.A., took a tough stance against Iraqi explanation that no Iraqi troops invaded Kuwait. It is said that the U.S.A. had continuously watched that Iraqi troops had been moving toward Kuwait. The U.S.A. succeeded in detecting the movement of some Iraqi soldiers based on computer-processed infrared data, which were obtained live via satellite. If several Iraqi soldiers can be identified by a satellite, it should be possible to make a diagnosis at a cellular-level through endoscopy. Thus, we began to investigate infrared rays as we developed an interest in it.

CHARACTERISTIC OF INFRARED RAYS

Infrared rays show high permeability, with which Iraqi soldiers were identified even under clouds. Such a characteristic of infrared rays has been applied to various technologies, such as endoscopy and non-destructive analysis of agricultural products. Infrared endoscope has been used for the observation of submucosal lesions and *in vivo* analysis of various substances. Unlike ultraviolet rays, infrared rays have no effects on DNA; they are benign rays for the human body. The most significant characteristic of infrared rays is that they produce no background noise, which is because of the absence of any substances emitting infrared fluorescence *in vivo*. Thus, if a diagnostic system using a substance labeled with infrared fluorescence is developed, diagnosis of a micro lesion becomes feasible. We therefore began to develop a diagnostic system for a microcancer using infrared fluorescence endoscopy.

INFRARED FLUORESCENCE SUBSTANCE

It was very difficult to find a substance labeled with infrared fluorescence, which can be used *in vivo*. However, we unexpectedly found such a substance in the field of ophthalmology, where ICG has been clinically utilized as an infrared fluorescence substance. Unfortunately, ICG cannot be used as a labeled substance because it has no protein-binding group. Thus, we began to synthesize an ICG derivative that has a protein-binding group. This is the beginning of our research in this area.

Finally we succeeded in synthesizing an ICG derivative (ICG-sulfo-Osu) with a protein binding group (Figure 1)(1). This derivative is a substance labeled with infrared fluorescence emitting 807 nm of fluorescence at 768 nm of excitation light (Figure 2).

INFRARED FLUORESCENT IMAGING SYSTEM

Thus, we experimentally produced an infrared fluorescent system using an excitation filter with transmission at 710-790 nm and a barrier filter with transmission at 810-920 nm (2, 3). We performed various basic examinations using this imaging system.

IMMUNOHISTOCHEMICAL STAINING

We treated a paraffin section of a gastric cancer tissue using an anti-CEA antibody labeled with the ICG derivative to carry out infrared fluorescent

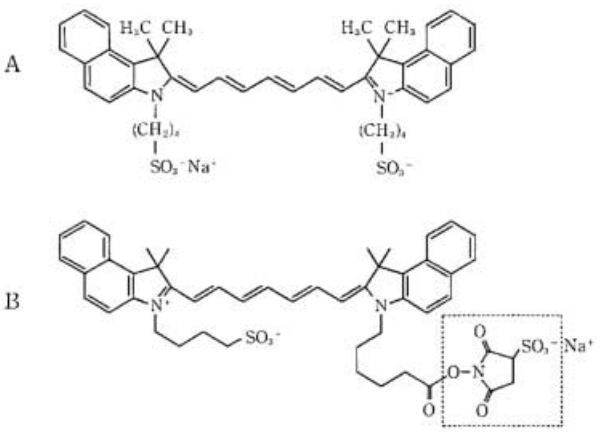

Figure 1. The chemical structure of ICG (A) and ICG-sulfo-OSu(B)

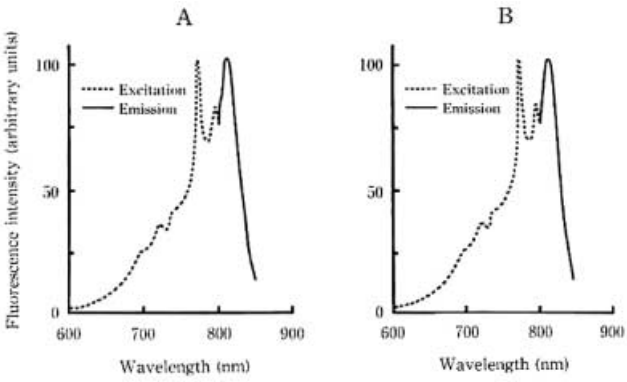

Figure 2. The excitation and emission spectrum of ICG (A) and ICG-sulfo-OSu (B)

observation. Although nothing was detectable under visible rays because the section was only treated with a labeled antibody, infrared fluorescence was seen on the lesion through infrared fluorescent observation (Figure 3)(4).

VITAL IMMUNOSTAINING

In vivo immunostaining is essential for this technique to be utilized in endoscopic diagnosis; however, no method has been established yet. Thus, we examined *in vivo* immunostaining using nude mice. A human gastric cancer was transplanted to the animals. The tumor was exposed under etherization. A tissue sample was collected after treated with an antibody. Immunostaining was performed using the ABC 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 at the luminary aspect of neoplastic glands. In positive controls, strong reactivity was seen, but in the negative control, reactivity was not observed (Figure 4)(5, 6).

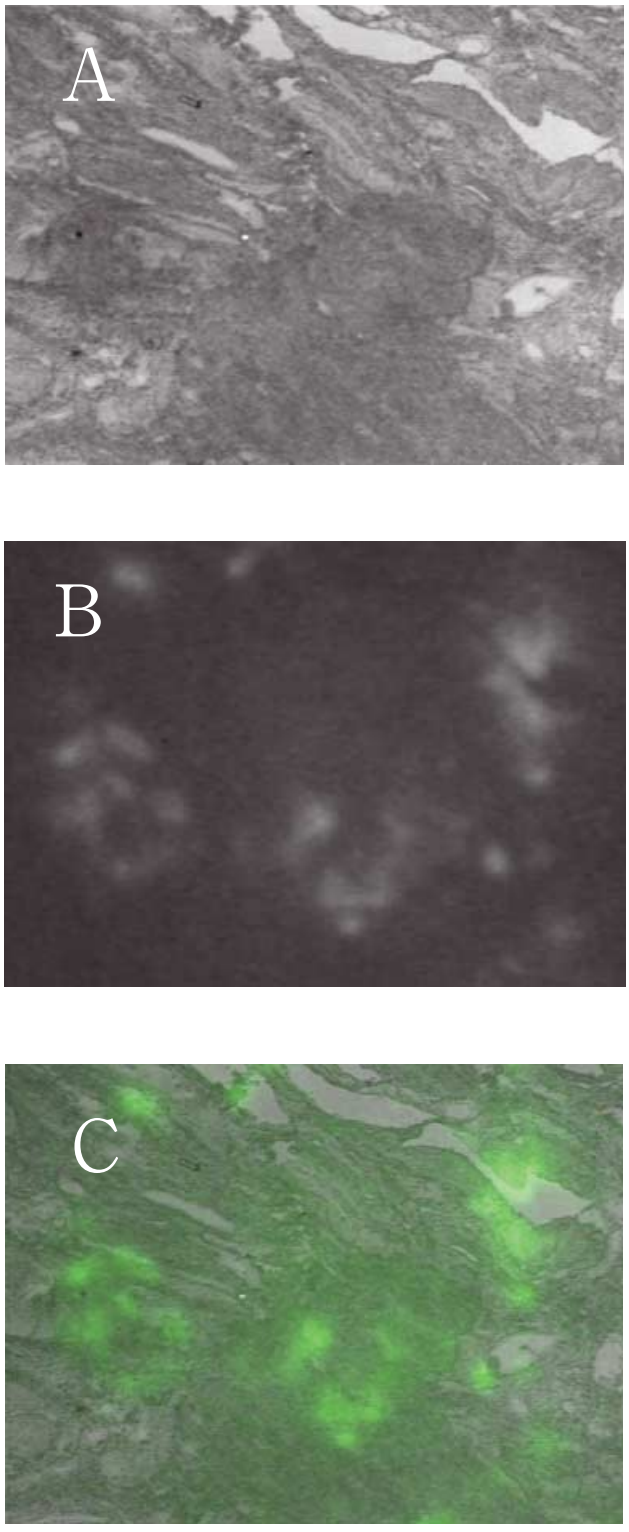

Figure 3. Infrared fluorescence imaging with ICG-sulfo-OSu labed anti-CEA antibody.
A : Image with visible rays B : Image with infrared fluorescence
C : Composite image

INFRARED FLUORESCENT ENDOSCOPE

Thus, we experimentally began to develop an infrared fluorescent endoscope with the same type of filters

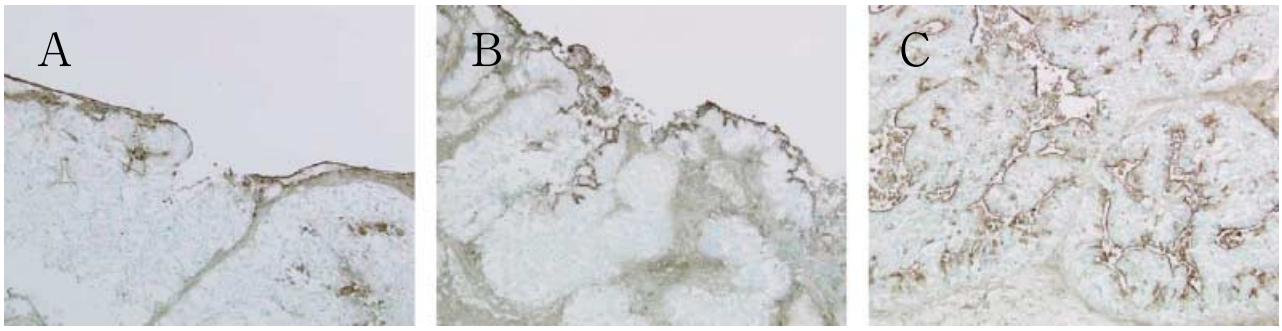

Figure 4. Vital immunostaining of human gastric cancers grafted into nude mouse.
A : Negative control B : Vital immunostaining
C : Conventional immunostaining

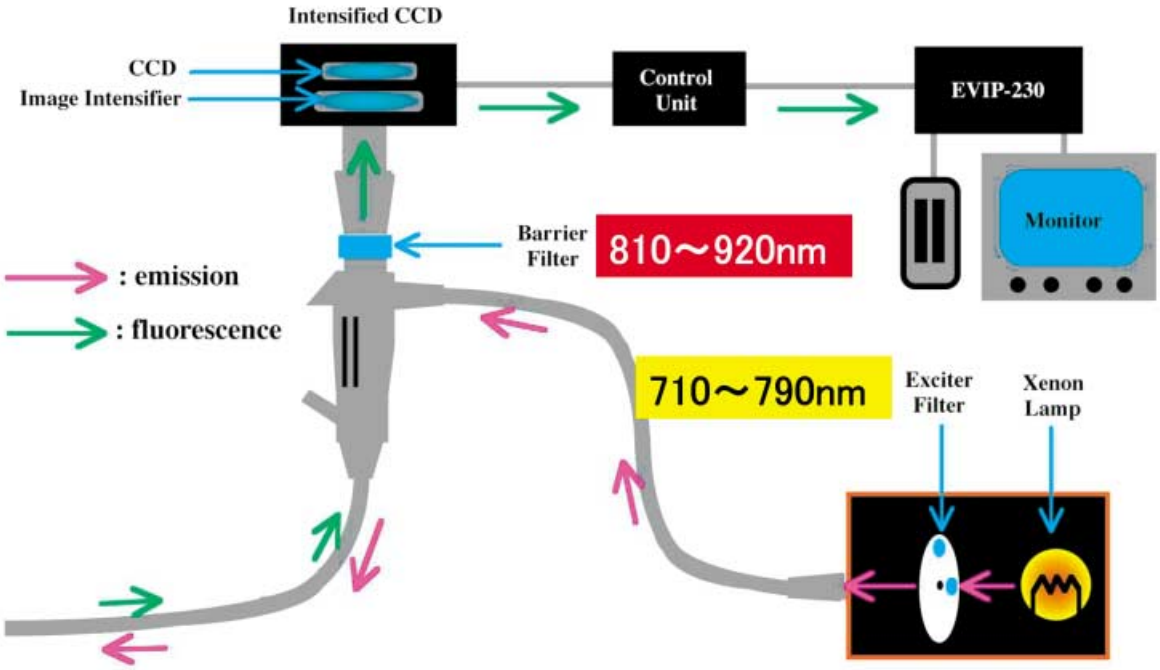

Figure 5. Infrared fluorescent endoscopy system

(Figure 5)(7). We performed an examination using a resected fresh specimen treated with an anti-CEA antibody labeled with an ICG derivative. After treating a resected fresh specimen with an anti-CEA antibody labeled with an ICG derivative, infrared fluorescent endoscopic images of a gastric cancerous lesion were taken. Infrared fluorescence was seen on the cancerous lesion (Figure 6)(7). When a lesion was removed and dyed with immunostaining according to the ABC method, color development of diamino benzidine(DAB) was observed on the site where infrared fluorescence was seen.

NEW ICG DERIVATIVE

Furthermore, we synthesized ICG-ATT as an ICG derivative with a stronger fluorescent intensity. The basic structure of ICG-ATT remains the same but the protein-binding group was different (Figure 7)(8). ICG and two types of ICG derivatives showed almost the same absorbance curve. As for fluorescence curve, there was little difference in the basic wavelength between ICG and two types of ICG derivatives although the wave form of ICG-ATT was slightly different (Figure 8, 9)(8). Figure 10 shows infrared fluorescence observed after treating a paraffin section with a labeled antibody. ICG-ATT showed slightly stronger fluorescence (9).

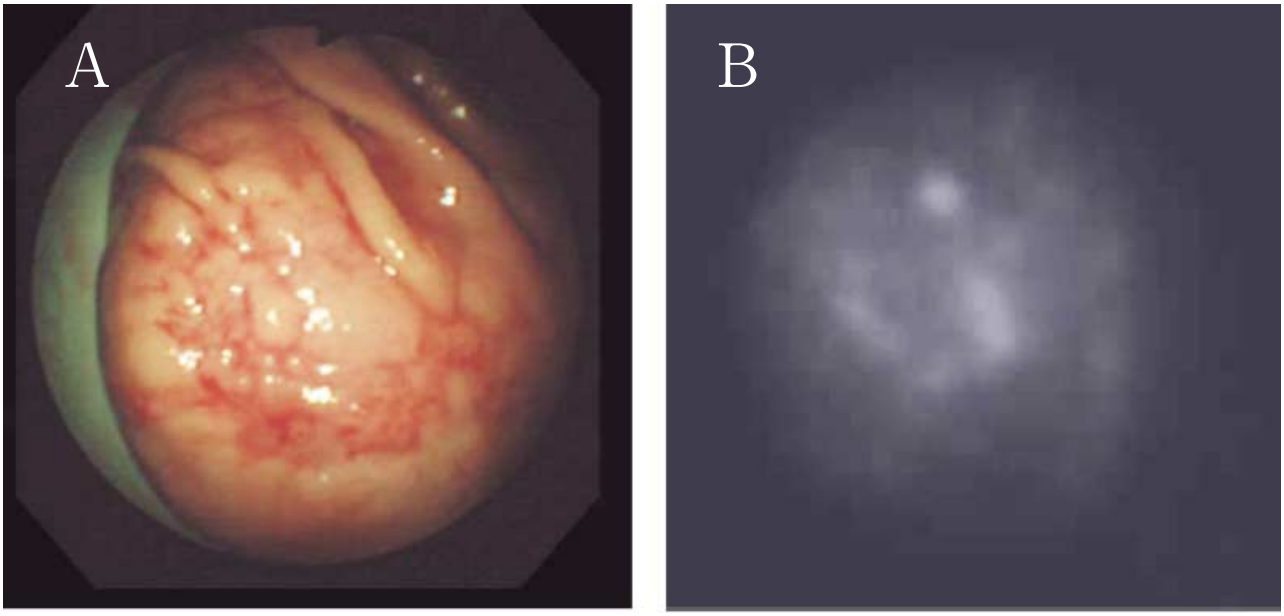

Figure 6. Infrared fluorescence imaging of freshly resected human gastric cancer tissue using ICG-sulfo-OSu labeled anti-CEA antibody.
A : Image with visible rays B : Image with infrared fluorescence

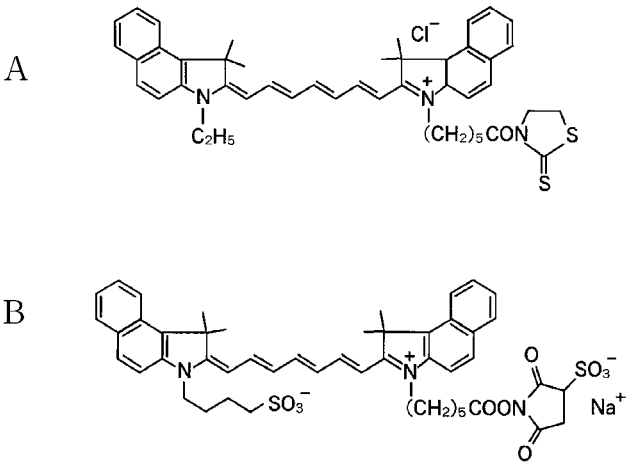

Figure 7. The chemical structures of ICG-ATT(A) and ICG-sulfo -OSu(B)

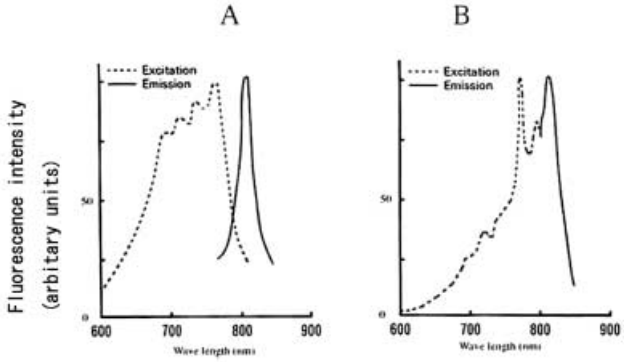

Figure 9. Fluorescent spectrum of ICG-ATT-(A) and ICG-sulfo -OSu-labeled anti-MUC1 antibodies (B).

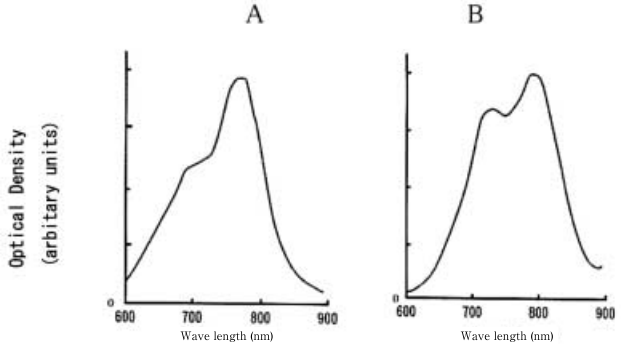

Figure 8. Absorption spectrum of ICG-ATT-(A) and ICG-sulfo -OSu-labeled anti-MUC1 antibodies(B).

REINFORCEMENT AGENTS

It was also found that some types of drugs (octylglucosid:OG) can be used as a reinforcement agent (10). With increasing concentration, the peak fluorescence wavelength lengthened;an increase in the OG concentration from 10 to 100mM resulted in a shift of peak fluorescence wavelength from 800 to 817nm (Figure 11)(10). Figure 12 shows infrared fluorescence images of sections treated with ICG-sulfo-OSu-MUC1-Ab solutions with or without OG. Without OG (Fig. 12 A), slight fluorescence was observed, however with 100 mM OG (Fig. 12 B), marked fluorescence was observed after image processing (10).

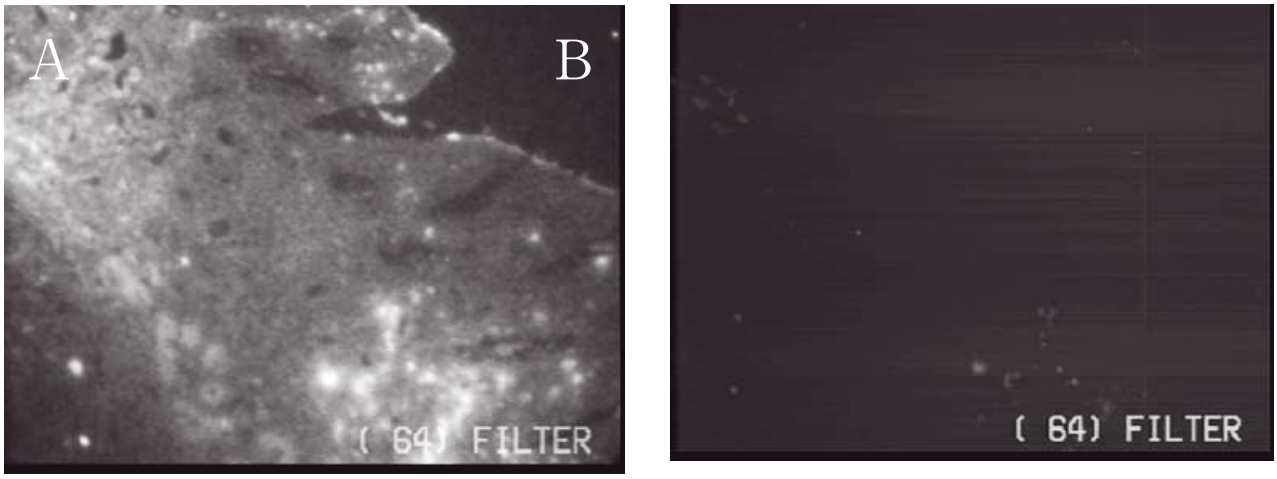

Figure 10. Infrared fluorescence observed after treating a paraffin section with ICG-ATT-(A) and ICG-sulfo-OSu-(B) labeled antibody.

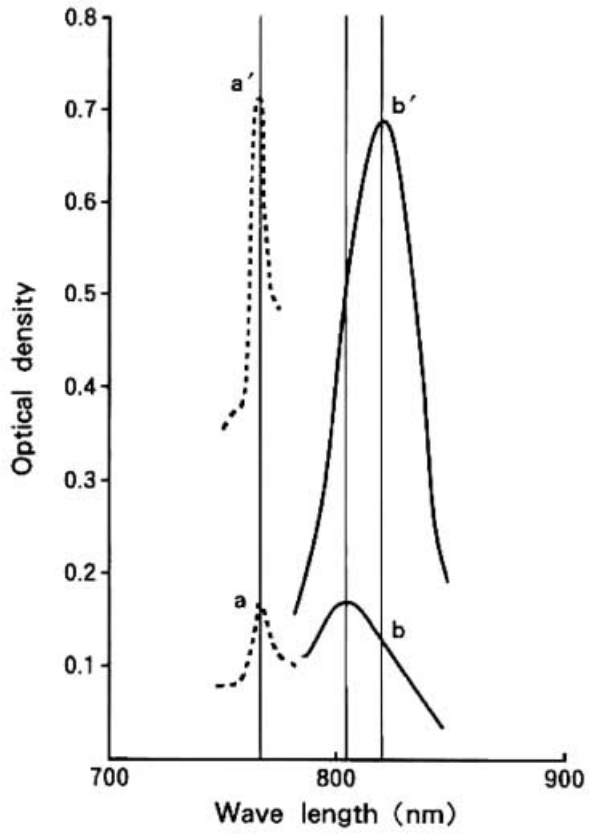

Figure 11. The excitation and emission spectra of ICG-sulfo-Osu labeled anti-MUC1 antibody before (a, b) and after(a',b') treated with reinforcement agent (ocutylglucoside) for fluorescence.
----- : excitation spectra, ——— : emission spectra

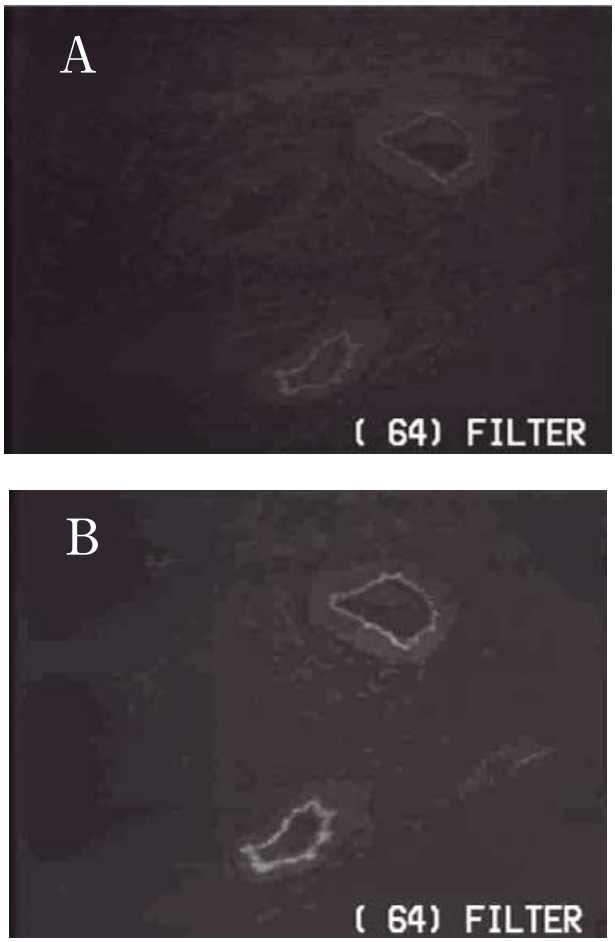

Figure 12. Infrared fluorescence image of sections treated with ICG-sulfo-OSu labeled anti MUC1 antibody with (B) or without(A) ocutylglucoside.

DISCUSSION

Although a diagnostic method of microcancer using *in vivo* immunostaining has not been established yet, an ICG derivative has been synthesized as a labeled substance that can be used *in vivo* and a reinforcement agent has successfully been developed. An infrared fluorescent endoscope has also been developed though experimentally. Although no immunostaining has been performed *in vivo*, the results of examinations using resected fresh specimens and nude mice indicate that *in vivo* immunostaining may be feasible. We believe that further investigation of cancer-specific antibodies will contribute to further progress in molecular biology.

As stated above, although a lot of problems remain

unsolved, we consider that they were at least partly solved.

As for *in vivo* immunostaining, Keller *et al,* in Germany successfully detected dysplasia in a model of ulcerative colitis using an existing substance labeled with fluorescence and a fluorescent endoscope(12). As for infrared fluorescence, the result of a study using cathepsin B-antibodies was reported (13). In the editorials in the same issue of the journal, titled " the coming revolution in gastrointestinal imaging, "it is written that bioendoscopy utilizing in vital immunostaining brought about a revolution in endoscopic diagnosis (14). The results of our study on infrared fluorescence were also mentioned in this editorial.

The progress of molecular biology is more remarkable than that of medical instruments. Human genome will soon be decoded and screening of various diseases based on genetic information will become available. We believe that the establishment of genetic diagnosis as a screening tool will have considerable influence on the course of medicine in the 21st century. Extremely high risk groups which have been screened in genetic diagnosis will be targeted. Thus, it will become essential to develop a system that can detect a very small lesion that is undetectable based on the currently available diagnostic systems or tools.

In the post-genome era, a screening system based on genetic diagnosis will be established and a new type of endoscopy targeting an extremely high risk population will become clinically available. Various types of new medical instruments will be developed and further advancement will be expected. We believe that the establishment of infrared fluorescent endoscopy will be an example of such progress in medicine.

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