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A New Infrared Fluorescent-Labeling Agent and Labeled Antibody for Diagnosing Microcancers

Masaya Tadatsu,[a] Susumu Ito,[a] Naoki Muguruma,[a,*] Yoshihiro Kusaka,[a] Kumi Inayama,[a] Terumi Bando,[a] Yoko Tadatsu,[a] Koichi Okamoto,[a] Kunio, Ii,[a] Yoshimitsu Nagao,[b] Shigeki Sano[b] and Hiromi Taue[b]

[a]*Department of Digestive and Cardiovascular Medicine, The University of Tokushima School of Medicine, 3-18-15, Kuramoto-cho, Tokushima City 770-8503, Japan*

[b]*Faculty of Pharmaceutical Sciences, The University of Tokushima School of Medicine, Tokushima, Japan*

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Abstract—Purpose: We have developed infrared fluorescent labeling agents and infrared-ray fluorescence endoscopes to establish a novel diagnostic technique. Since the fluorescence intensity of the initial labeled antibody (ICG-sulfo-OSu-labeled antibody) was not sufficient for practical use, we synthesized indocyanine green acylthiazolidinethione (ICG-ATT), which was expected to label various target molecules having amino groups efficiently. Materials and Methods: To confirm imaging of infrared fluorescence intensity of ICG-ATT- and ICG-sulfo-OSu-labeled anti-MUC1 antibodies, cotton thread was soaked in various concentrations of the antibody solution in 0.1 M PBS, and observed under the epi-illumination infrared fluorescence microscope. Localization and the intensity of infrared fluorescence and DAB coloring was compared in paraffin sections of human gastric mucosa. Results: In the study of cotton threads, both labeled antibodies showed relatively clear infrared fluorescence, and significant difference was not observed between the two antibodies. ICG-ATT-labeled anti-MUC1 antibody produced stronger staining than that by ICG-sulfo-OSu-labeled antibody. Localization pattern of infrared fluorescent staining was in good agreement with that by the conventional method with oxidized DAB staining. Conclusion: ICG-ATT is useful as a fluorescent-labeling agent for diagnosis of microcancers by infrared fluorescence endoscopes.

Introduction

We have developed infrared fluorescent labeling agents and infrared-ray fluorescence endoscopes to establish a novel diagnostic technique for microcancers by utilizing advantageous functions of electronic endoscopes. First, we synthesized an indocyanine green (ICG) derivative, indocyanine green *N*-hydroxysuccinimide ester (ICG-sulfo-OSu),[1] and developed an infrared fluorescence imaging device.[2,3] An antibody labeled with this ICG derivative was shown to maintain the antibody reactivity and to emit fluorescence by near-infrared radiation,[4] and detected human gastric cancer in resected specimens using ICG-sulfo-Osu labeled anti-human carcinoembryonic antigen antibody with an infrared fluorescence endoscopy.[5] However, the fluorescence intensity of the antibody was not sufficient for practical use, even in the combination of an agents for reinforcement of fluorescence.[6,7] Therefore, we next synthesized indocyanine green acylthiazolidinethione (ICG-ATT), which was expected to label various target molecules having amino groups efficiently.[8] ICG-ATT consists of the asymmetric ICG skeleton, alkyl side chain and the thiazolidinethioneamide (ATT) group, which has been developed by Nagao et al.[8] The compound shows strong absorption derived from ICG at the near-infrared range around 800 nm, and the ATT moiety can react with amine residues of proteins, resulting in covalent labeling of target proteins. These characteristics of ICG-ATT have suggested that this compound can be useful for infrared fluorescent labeling, as well as ICG-sulfo-OSu. However, comparative study on fluorescence intensity of antibodies labeled with these ICG derivatives has not been performed.

In this study, we compared spectroscopic characteristics and fluorescence intensity of ICG-ATT- and ICG-sulfo-OSu-labeled antibodies.

*Corresponding author. Tel.: +81-88-633-7124; fax: +81-88-633-9235; e-mail: muguruma@clin.med.tokushima-u.ac.jp

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Materials

The infrared fluorescent labeling agent, ICG-ATT (MW = 760.50), was synthesized by Nagao et al.,[8] and ICG-sulfo-OSu (MW = 930.09) was purchased from Dojindo Laboratories. An antibody (MY.1E 12)(MW about 150,000, prepared to 3.1 mg/mL PBS) against a mucin glycoprotein, MUC1, was a generous gift from Prof. T. Irimura (The University of Tokyo).[9] Labeling of the antibody with ICG-ATT and ICG-sulfo-OSu was performed according to the previous method.[4] Normal horse serum was included in the Vectastain ABC-PO (mouse IgG) kit (Funakoshi Co., Ltd.). Paraffin sections of the gastric mucosal tissue, which had been proved to be positive in anti-MUC1-antibody staining, were used in immunohistochemistry.

Measurement of absorption and fluorescence spectra was performed on DU650 spectrophotometer (Beckman Coulter, Inc.) and 650-40 spectrofluorometer (Hitachi Instruments, Inc.). An epi-illumination infrared fluorescence microscope (OLYMPUS OPTICAL Co., Ltd.) was used for infrared fluorescence imaging, according to the previous report.[2]

Methods

Measurement of absorption and fluorescence spectra of the infrared-fluorescent-labeled antibodies

ICG-ATT solution (828 μmol/L) was prepared by dissolving 1.26 mg of ICG-ATT in 2 mL of dimethylformamide (DMF). ICG-sulfo-OSu solution was prepared from 1.54 mg of ICG-sulfo-OSu in the similar manner. Absorption and fluorescence spectra of the two compounds were analyzed.

To label the antibody, 100 μL of ICG-ATT solution (0.0828 μmol, 4-mol equivalent to the antibody) was added to 1 mL of anti-MUC1 antibody solution (0.0207 μmol). The mixture was kept at room temperature in the dark for 1.5 h, and then loaded onto a PD-10 column (Amersham Pharmacia Biotech, Inc.) after adding 1.4 mL of 0.1 M phosphate-buffered saline (pH 7.5, PBS) to a final volume of 2.5 mL. The labeled antibody was eluted as a green-colored fraction with 3.5 mL of PBS, and lyophilized. ICG-ATT- and ICG-sulfo-OSu-labeled anti-MUC1 antibodies thus obtained were diluted to 0.5, 5.0, 50 and 500 μg/mL in 0.1 M PBS (pH 7.5)/DMF (9/1), which were subjected to analyses of absorption and fluorescence spectra.

Confirmation of antibody labeling by infrared fluorescence imaging using cotton thread

To confirm imaging of infrared fluorescence intensity of ICG-ATT- and ICG-sulfo-OSu-labeled anti-MUC1 antibodies, cotton thread was soaked in various concentrations (0.5, 5.0, 50 and 500 μg/mL) of the antibody solution in 0.1 M PBS, and observed under the epi-illumination infrared fluorescence microscope.[3] ICG-ATT and ICG-sulfo-OSu were also examined at 0.1, 1.0, 10 and 100 μg/mL in 0.1 M PBS in the same manner. Fluorescence intensity was exhibited by − (no fluorescence), + (weak fluorescence detected), ++ (moderate fluorescence) and +++(clear fluorescent images obtained).

Immunohistochemistry

Paraffin sections of human gastric mucosa, which had been previously proven to be positive in usual immunostaining for the anti-MUC1 antibody, were deparaffinized, and xylene was removed. After blocking endogenous peroxidase activity, sections were incubated with normal horse serum for 20 min, and then with the labeled antibodies at 0.5, 5.0, 50 and 500 μg/mL in 0.1 M PBS for 10 min at room temperature. Observation was performed under the infrared fluorescence imaging device to compare the fluorescence intensity of each preparation. Adjacent sections processed in the same way were incubated with the primary antibody, and then treated with the secondary antibody and the ABC reagent (avidin biotinylated peroxidase complex) for 30 min. Preparations were visualized by 3-3′ diaminobenzidine (DAB), and counter-stained by hematoxylin and methyl green. Localization of infrared fluorescence and DAB coloring was compared in serial sections.

Results

Characteristics of absorption and fluorescence spectra of the infrared-fluorescent-labeled antibodies

Table 1 shows maximal wavelength for absorption, excitation and fluorescence of ICG-ATT, ICG-sulfo-OSu, ICG-ATT- and ICG-sulfo-OSu-labeled anti-MUC1 antibodies in PBS. Absorption maxima of ICG-ATT and ICG-sulfo-OSu were both 783 nm. Maximal wavelength for excitation and fluorescence of ICG-ATT was 768 and 804 nm, respectively, which were similar to those of ICG-sulfo-OSu. Both ICG-ATT- and ICG-sulfo-OSu-labeled anti-MUC1 antibodies showed the same maximal wavelength for absorption, excitation and fluorescence at 786, 766, and 804 nm, respectively. The maximal absorption of the antibodies at 786 nm showed a slight red-shift from that of ICG-ATT and ICG-sulfo-OSu, 783 nm. The maximal excitation wavelength was also shifted from 768 to 766 nm, but no other significant change was observed.

Absorption, excitation and fluorescence spectra of the labeled antibodies are shown in Figures 1 and 2. The

Table 1. Spectroscopic characteristics of ICG derivatives and ICG-derivatives and ICG-derivative-labeled antibodies in fluorescence analysis

ICG derivatives	Absorption (nm)	Excitation (nm)	Emission (nm)
ICG-sulfo-OSu (10 μg/mL)	783	768	804
ICG-ATT (10 μg/mL)	783	768	804
ICG-sulfo-OSu-MUC1 (500 μg/mL)	786	766	804
ICG-ATT-MUC1 (500 μg/mL)	786	766	804

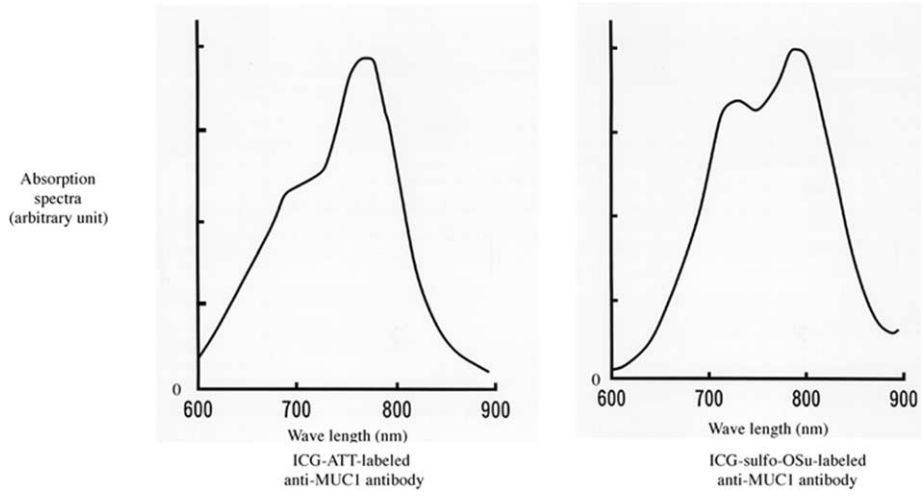

Figure 1. Absorption spectra of ICG-ATT- and ICG-sulfo-OSu-labeled anti-MUC1 antibodies. (A) Absorption spectra of ICG-ATT-labeled anti-MUC1 antibodies. (500 μg/mL). (B) Absorption spectra of ICG-sulfo-OSu -labeled anti-MUC1 antibodies. (500 μg/mL).

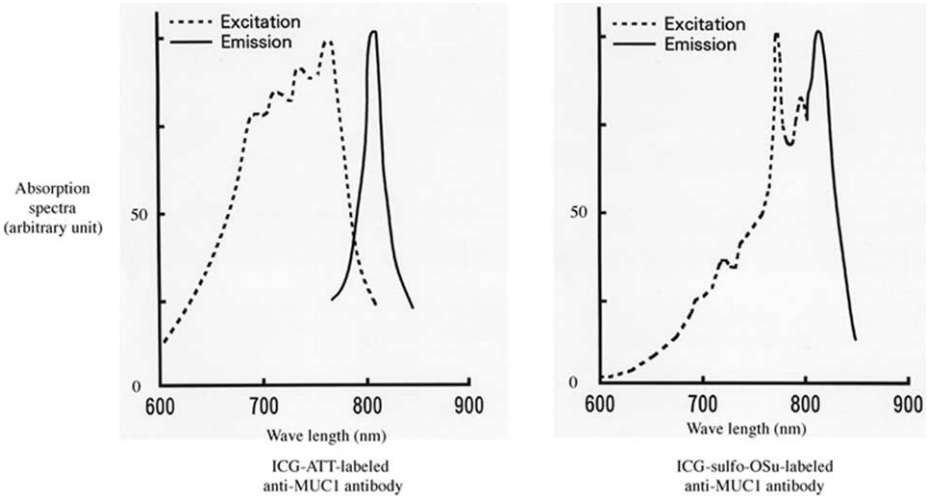

Figure 2. Excitation and fluorescence spectra of ICG-ATT- and ICG-sulfo-OSu-labeled anti-MUC1 antibodies. (A) Excitation and fluorescence spectra of ICG-ATT- labeled anti-MUC1 antibodies. (500 μg/mL). (B) Excitation and fluorescence spectra of ICG-sulfo-OSu - labeled anti-MUC1 antibodies (500 μg/mL).

absorption spectra of ICG-ATT- and ICG-sulfo-OSu-labeled antibodies exhibited the similar pattern, showing the strongest absorption at 786 nm (Fig. 1). In the excitation and fluorescence spectral patterns (Fig. 2), slight difference was observed between the two labeled antibodies, but it was demonstrated that both were excited at 766 nm in the near-infrared range, and emitted the strongest fluorescence at 804 nm, which was well consistent with the results of the two fluorescent dyes.

Table 2 demonstrates the comparison of fluorescence intensity of ICG-ATT, ICG-sulfo-OSu, and the antibodies labeled with these substances. ICG-ATT and ICG-sulfo-OSu showed the strongest fluorescence at the concentrations of 1.0 and 10 μg/mL, respectively. When comparing at the same concentrations, the fluorescence of ICG-sulfo-OSu was stronger than that of ICG-ATT. Both of the labeled antibodies emitted the strongest fluorescence at 500 μg/mL, at which the intensity of the two antibodies was similar to each other.

Confirmation of antibody labeling by infrared fluorescence imaging using cotton thread

Results of infrared fluorescent observation of the cotton thread soaked in various sample solutions are summarized in Table 3. The clearest images for ICG-ATT and ICG-sulfo-OSu were obtained at 10 and 100 μg/mL, respectively. In the case of labeled antibodies, the strongest fluorescence was obtained at 500 μg/mL. Figure 3 shows examples of infrared fluorescence images of the cotton thread soaked in ICG-ATT- and ICG-sulfo-OSu-labeled antibody solutions (500 μg/mL). Both preparations showed relatively clear infrared fluorescence, and significant difference was not observed between the two antibodies.

Immunohistochemistry

Immunohistochemical staining of the paraffin sections of human gastric mucosa by infrared fluorescence and

Table 2. Fluorescence of ICG derivatives and ICG-derivative-labeled antibodies

ICG derivatives	Concentration (μg/mL)	Intensity of fluorescence (A.U.)
ICG-sulfo-OSu	0.1	15.5
	1.0	157.0
	10.0	250.0
	100.0	N.D.
ICG-ATT	0.1	7.36
	1.0	55.0
	10.0	22.7
	100.0	2.83
ICG-sulfo-OSu-MUC1	0.5	N.D.
	5.0	N.D.
	50.0	2.32
	500.0	24.4
ICG-ATT-MUC1	0.5	N.D.
	5.0	1.24
	50.0	7.33
	500.0	27.4

A.U.: Arbitrary unit, N.D.: No data.

Table 3. Fluorescence intensity of cotton threads in infrared fluorescence imaging

ICG derivatives	Concentration (μg/mL)	Intensity of fluorescence (A.U.)
ICG-sulfo-OSu	0.1	−
	1.0	+
	10.0	+ +
	100.0	+ + +
ICG-ATT	0.1	−
	1.0	+ +
	10.0	+ + +
	100.0	+ +
ICG-sulfo-OSu-MUC1	0.5	−
	5.0	−
	50.0	+
	500.0	+ +
ICG-ATT-MUC1	0.5	−
	5.0	−
	50.0	+
	500.0	+ +

conventional DAB coloring is shown in Figure 4. ICG-ATT-labeled anti-MUC1 antibody (Fig. 4a) produced stronger staining than that by ICG-sulfo-OSu-labeled antibody (Fig. 4b). Localization pattern of infrared fluorescent staining was in good agreement with that by the conventional method with oxidized DAB staining, which confirmed that the fluorescence due to the ICG-ATT-labeled antibody represented the antigen-antibody reactions for MUC1 (Fig. 4c).

Discussion

Many efforts have been made to recognize microcancer lesions by employing electronic endoscopes,[10] and two major research directions have been proposed. One includes the method, in which images of microlesions are obtained by utilizing differences in color tones and fluorescence between the lesions and normal regions and by improving imaging devices, without any processing of the lesions themselves. There has been a report employing self-fluorescence.[11] In the other method,

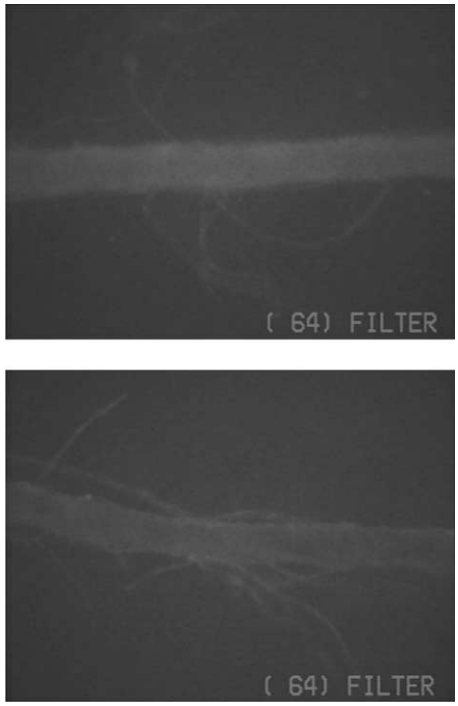

Figure 3. Infrared fluorescence images of cotton thread soaked in ICG-ATT-labeled anti-MUC1 antibody (A) and in ICG-sulfo-OSu-labeled anti-MUC1 antibody (B). Concentration of the antibody solutions was 500 μg/mL in both cases.

microlesions are labeled with chemicals, and electronic signals from the labeling compounds are used for imaging. Various fluorescent dyes that emit fluorescence in the ultraviolet range have been developed for this method.[12,13] However, the fluorescent chemicals so far developed show strong cytotoxicity[14] or are injurious to cells due to ultraviolet irradiation,[15] which prevents practical use of the compounds for observation of vital lesions. Moreover, background noise cannot be ignored in the ultraviolet and visible ranges, which might interfere clear observation. In contrast, observation in the near-infrared range is not affected by background noise,[16] and there are little adverse effects on vital tissue. Thus, chemicals that emit fluorescence in the near-infrared range and have low toxicity are thought to be suitable as labeling agents. Additionally, in practical immunohistochemical staining, direct labeling is preferable to indirect one, because the indirect method requires longer reaction time than that for direct labeling, which often results in non-specific staining. To achieve this, high specificity of primary antibodies as well as high sensitivity of fluorescent-labeling agents is essential.[17] In the previous reports, we focused on ICG, which had been widely used in the clinical field[18,19] as a dye emitting fluorescence in the near-infrared range, and labeled the anti-epithelial-membrane-antigen (EMA) antibody with ICG-sulfo-OSu that possessed the ICG skeleton. We attempted to use the labeled antibody for immunohistochemical staining of the esophageal mucosa, but the fluorescence intensity was not sufficient.[4] Therefore, we investigated the possibility of ICG-ATT as an alternative fluorescent-labeling agent in the present study.

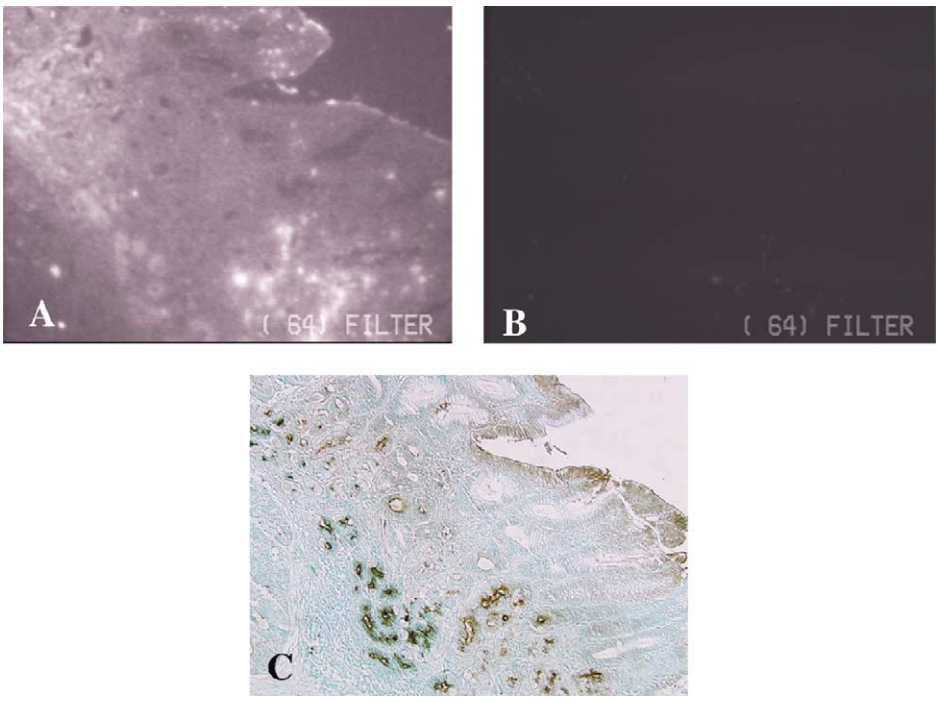

Figure 4. Immunohistochemical staining of the paraffin sections of human gastric mucosa. (A) Staining with ICG-ATT-labeled antibody (500 μg/mL) under excitation by infrared rays. (B) Staining with ICG-sulfo-OSu -labeled antibody (500 μg/mL) under excitation by infrared rays. (C) Staining with the conventional ABC method with DAB coloring.

ICG-ATT is a novel fluorescent-labeling agent originally developed by Nagao et al. in 1998.[8] The compound is characterized by; (1) showing strong absorption at 783 nm in the near-infrared range, (2) having the thiazolidineamide (ATT) group capable of specific acylation of amino groups, and (3) being an analogue of ICG that has been established as a safe diagnostic agent for hepatic functions. Furthermore, compared to the fluorescent dyes used in conventional antibody labeling methods, which show absorption in the ultraviolet and visible ranges, ICG-ATT is advantageous because it is not affected by contaminant components and/or solvent interference in fluorescence analysis.[16] In addition to these points, fluorescent agents used for antibody labeling should have a sufficiently large molar extinction coefficient ($\geq$100,000 in organic compounds), which is an index for absorption intensity, the maximal excitation wavelength should be sufficiently longer than that for aromatic amino acids (280 nm), and there should be a sufficient shift between the maximal excitation wavelength and the maximal fluorescence wavelength (Stoke's shift). To examine whether ICG-ATT satisfies these requirements, we analyzed absorption and fluorescence spectra of the compound, and calculated the molar extinction coefficient in this study. The maximal wavelength for absorption was 783 nm, and the molar extinction coefficient was about 140,000, indicating that ICG-ATT showed strong absorption derived from a long coupling structure of ICG in the near-infrared range. The maximal wavelength for excitation and fluorescence was 766 and 804 nm, respectively, and the Stoke's shift was relatively large (38 nm), which supports the notion that ICG-ATT could be a useful fluorescent-labeling agent for antibody labeling.

When fluorescent-labeling agents are conjugated with proteins such as antibodies, characteristics of absorption and fluorescence spectra of the agents might be changed. Therefore, we measured absorption and fluorescence spectra of the ICG-ATT-labeled antibody. The results demonstrated that the labeled antibody was successfully excited and emitted fluorescence, showing the similar spectral pattern to that of the fluorescent dye (ICG-ATT), confirming that the labeled antibody maintained fluorescence characteristics of the ICG dye. In immunohistochemistry, fluorescence due to the ICG-ATT-labeled antibody was observed in good agreement with the antigen-antibody reaction, suggesting that the ICG-ATT-labeled antibody is useful for fluorescent labeling.

We also compared spectroscopic characteristics of ICG-ATT- and ICG-sulfo-OSu-labeled antibodies. The two antibodies showed the similar fluorescence intensity at the same concentrations, but the ICG-ATT-labeled antibody gave much stronger fluorescence when reacted with the antigen on the tissue sections. This might be because a decrease in antigen affinity of the antibody caused by dye coupling was smaller in ICG-ATT labeling. Reasons for decreased affinity of labeled antibodies could be; (1) a fluorescent-labeling agent is coupled to the antigen-binding site of the primary antibody, (2) the tertiary structure of the antibody is markedly changed by binding of the antibody and a labeling compound, (3) polarization of electric charge is caused by binding of the antibody and a labeling compound, (4) the antigen-antibody reaction is competitively inhibited by unlabeled antibodies coexisting with labeled ones, and (5) the labeled antibody is non-covalently reacted with the antigen. Which reason most affects difference in

staining remains unknown. In the case of (3), there is the possibility that the charge state is different between ICG-ATT and ICG-sulfo-OSu, since the two substances have different carbon chains connected to the ICG skeleton. The terminal side chain of ICG-sulfo-OSu is negatively charged, which might affect the charge of the whole antibody protein to induce non-specific binding of the antibody to the surrounding tissue by static electric power. As a result, competitive inhibition on the antigen-antibody reaction is caused, resulting in decreased staining of the specific reaction. As to (2), the planar structure of the ICG skeleton is important for expression of fluorescence. If the charge state was different between the two compounds and ICG-sulfo-OSu undergoes larger change in the planar structure, the change might prevent fluorescence expression of ICG-sulfo-OSu in the antigen-antibody reaction. Furthermore, (5) raises the possibility that non-covalent binding occurs on the ICG skeleton, resulting in difference in covalent/non-covalent binding ratio between the two labeled antibodies. However, detailed mechanisms for the decreased antigen affinity and difference in non-specific staining were unclear. The molar ratio for binding of ICG-ATT and the primary antibody, and the ratio of covalent/non-covalent binding should be clarified in the future work.

In conclusion, the comparative study on infrared fluorescence and immunohistochemical staining for ICG-ATT- and ICG-sulfo-OSu antibodies demonstrated that the ICG-ATT-labeled antibody gave stronger fluorescence in the tissue preparations than the ICG-sulfo-OSu-labeled one, suggesting that ICG-ATT is useful as a fluorescent-labeling agent for diagnosis of microcancers by infrared fluorescence endoscopes.

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