Labeled Carcinoembryonic Antigen Antibodies Excitable by Infrared Rays: A Novel Diagnostic Method for Micro Cancers in the Digestive Tract

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Object An indocyanine green derivative (ICG-sulfo-OSu) was used as the labeling substance for monoclonal antibody, and a fluorescence imaging system appropriate for ICG-sulfo-OSu excitable by infrared rays (IR) was developed. The goal of this study was to demonstrate antibody labeling at the tissue level using this new imaging system. Materials and Methods ICG-sulfo-OSu labeled mouse anti-human carcinoembryonic antigen (CEA) monoclonal antibody, a newly developed imaging system, and an infrared ray microscope were employed in this experiment. Paraffin sections of human colon cancer previously proven to have cross-reactivity to anti-CEA antibody were examined. Results Positive staining was seen as a brownish discoloration of oxidized 3,3′-diaminobenzidine tetrahydrochloride (DAB) in sections that reacted with ICG-sulfo-OSu-labeled anti-CEA antibody, and the fluorescence was well-matched with the oxidized DAB-positive sites. Conclusion Specific antibodies labeled with ICG-sulfo-OSu have significant affinity to cancer cells and seem to reflect sufficient amounts of fluorescence by IR to be useful in a system for the endoscopic detection of micro cancers using the immunohistochemical staining method.

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Key words: labeled monoclonal antibody, infrared ray fluorescence, immunofluorescence, infrared ray microscope

Introduction

Recently, attempts have been made to use monoclonal antibodies to develop methods of diagnosis and treatment that are highly specific to particular target tissues (1–3). In the field of gastroenterology (4, 5), attempts have been made to check for lesions using carcinoembryonic antigen (CEA) antibody labeled with fluorescent material. However, since the wavelength of fluorescence used in those studies was in the ultraviolet range, its use in vivo is limited due to safety concerns (6) and due to interference from some other sources of fluorescence (7). To resolve these problems, we have developed a labeling substance that emits a particular wavelength of fluorescence when excited by near-infrared rays. In the present study, human colorectal cancer tissue specimens were treated with labeled CEA antibodies for observation using this recently developed infrared fluorescence imaging system. The fluorescent images obtained were analyzed.

Materials and Methods

Materials

Indocyanine green derivative (ICG-sulfo-OSu; Daiichi Pure Chemicals Co., Tokyo) was used in this experiment. The physiochemical character of this labeling substance resembles that of ICG, but ICG-sulfo-OSu differs from ICG in that it has an active ester group, which is a characteristic chemical struc-
ture capable of binding to various antibodies (Fig. 1, squared) (8). And this labeling substance has a specific fluorescence emission at 807 nm upon excitation at 768 nm (Fig. 2) (8). Mouse anti-human CEA monoclonal antibody was obtained from Chemicon International Inc., CA, USA (Lot number 31696285). CEA antibody was labeled with ICG-sulfo-OSu as reported previously (9).

According to standardized procedure, paraffin sections of 30 μm thickness obtained from 10 cases of colon cancer were stained with anti-CEA antibody by avidin-biotinylated peroxidase complex (ABC) method. Five specimens stained with brownish discoloration of oxidized 3,3′-diaminobenzidine tetrahydro-chloride (DAB), suggesting cross reactivity to human CEA, were used for the IR imaging analysis. Normal human skeletal muscle as a control was also examined.

**Imaging system**

We previously studied on infrared fluorescent images of gastric carcinoma that were obtained with a transmitted-type imaging system (Ito S et al, unpublished observation). The present study was designed to develop a method of endoscopic diagnosis using labeled monoclonal antibodies. The transmitted-type system, in which the specimen is sandwiched between the barrier filter and exciter filter, cannot be easily used for endoscopy because excitation of the sample and observation of fluorescence must be performed on the same side of the

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**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).
sample during endoscopic observation. For application of infrared fluorescence endoscopy, we developed a new reflected-type imaging system and used it in this study (Taoka S et al, unpublished observation).

Figure 3 shows the reflected-type imaging system for labeled substances excited by near-infrared rays. An infrared microscope for visible to near-infrared rays (BX-60, Olympus Optical Co., Ltd., Tokyo) was used. To develop the reflected type infrared fluorescence imaging system, the light source was placed in the microscope, and samples were irradiated using a half mirror. The exciter filter (transmission wavelengths: 710–790 nm) was placed between the halogen lamp and the half mirror, and the barrier filter (transmission wavelengths: 810–920 nm) was placed between the half mirror and the ICCD camera (ICCD-500/DF, Hamamatsu Photonics, Japan). Fluorescence signals were detected with the ICCD camera, captured with an image capturing device (EVIP-230, Olympus Optical Co., Ltd.), and recorded on an image storage device (230MO Turbo II+, Olympus Optical Co., Ltd.). Light from the light source passes through the exciter filter, and about 50% of the light is reflected and irradiates the samples.

**Observation of tissue sections under infrared fluorescence imaging system**

Sections on the slide glass were immersed in 0.3% hydrogen peroxide-methanol for 30 minutes in order to inactivate endogenous peroxidase activity, followed by rinsing 3 times with 0.01 M phosphate-buffered saline (PBS) for 5 minutes each time. Then each specimen was treated with 2% horse serum for 15 minutes at room temperature. After rinsing with PBS, the specimen was incubated with ICG-sulfo-OSu-labeled anti-CEA antibody for 90 minutes at room temperature, and infrared rays (IR) imaging analysis was performed. The antibody was diluted with 0.01 mM PBS containing 0.5% bovine serum albumin and the optimal dilution was 1:25, as shown in our previous study (9).

**Results**

**Immunohistochemical demonstration of CEA using ICG-sulfo-OSu labeled anti-CEA antibody**

ICG-sulfo-OSu labeled anti-CEA antibody was examined in 5 specimens of human colon cancer and normal human skeletal muscle. The immunoreactive stainings with ICG-sulfo-OSu labeled anti-CEA antibody were confirmed only in sections of human colon cancer as brownish discoloration of oxidized DAB but not in the normal skeletal muscle (Table 1).

Figure 4 shows the prepared colon cancer specimen (moderately differentiated tubular adenocarcinoma) treated by hematoxylin and eosin staining (A) and immunohistochemical staining with ICG-sulfo-OSu labeled anti-CEA antibody (B).

**Observation of tissue sections using the infrared fluorescent imaging system**

Figure 5 shows the IR fluorescence images of a colon cancer specimen (moderately differentiated tubular adenocarcinoma) treated with ICG-sulfo-OSu labeled anti-CEA antibody, as seen under visible rays (A) and IR fluorescence (B). IR fluorescence was observed in the colon cancer but not in skeletal muscle. The sites where IR fluorescence was observed corresponded to the DAB-positive sites disclosed by conventional immunohistochemical staining. No fluorescence was observed in control sections (Table 1), suggesting that the observed fluorescence was considered to be due to immunohistochemical reaction with the ICG-sulfo-OSu labeled antibodies.

**Discussion**

Endoscopic diagnosis has advanced remarkably following the development of the electronic endoscope. However, morphological diagnosis of micro-lesions using electronic endoscopes has limitations, so a biopsy is still needed to confirm diagnosis. In recent years, various attempts have been made...
Table 1. Immunofluorescent Images with ICG-sulfo-OSu Labeled Anti-CEA Antibody in Colon Cancer Sections Compared with the ABC Method

<table>
<thead>
<tr>
<th>Case</th>
<th>Histology of tissue</th>
<th>ABC method with ICG-sulfo-OSu labeled anti-CEA antibody</th>
<th>IR fluorescence image</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>Well differentiated tubular adenocarcinoma</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Case 2</td>
<td>Moderately differentiated tubular adenocarcinoma</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Case 3</td>
<td>Moderately differentiated tubular adenocarcinoma</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Case 4</td>
<td>Moderately differentiated tubular adenocarcinoma</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Case 5</td>
<td>Poorly differentiated tubular adenocarcinoma</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td>Normal human skeletal muscle</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ABC: avidin-biotinylated peroxidase complex.

Figure 4. Prepared specimen of human colon cancer. A) HE stain (×66). B) Immunohistochemical staining by ABC method (CEA, monoclonal, 1:25) (×66).
to obtain images that reflect the histopathological features of lesions. Several attempts using labeled CEA antibodies have also been reported (2). Fluorescence that is specific to gastrointestinal lesions has been observed in studies using isolated specimens (4, 5). However, the use of fluorescence of ultraviolet-range wavelength results in interference from strong background noise (7) and possible damage to the living tissue (6). To overcome these problems, infrared-range wavelengths are preferable because they produce less noise and are safer for this kind of diagnostic system.

ICG, which is often used as an indicator of liver function (10), emits fluorescence when excited by infrared rays (11). ICG, however, does not bind to protein. Therefore, it is not possible to label monoclonal antibodies with ICG. We have recently developed ICG-sulfo-OSu, which can bind to protein (8), and we have labeled various antibodies with ICG-sulfo-OSu (9). Furthermore, we have developed an imaging system that detects the radiation of particular wavelengths produced when antibodies labeled with this agent are excited by certain wavelengths of light (12). With this system, a specimen is sandwiched between an excitation filter and a barrier filter. This transmitted-type system, however, is difficult to apply to an endoscopic system. In the present study, which was designed to develop a system for endoscopic diagnosis using labeled monoclonal antibodies, we used a recently developed, reflected-type of infrared fluorescence imaging system (Taoka S et al, unpublished observation).

When paraffin sections of human colorectal carcinoma were observed with this system, fluorescence was detected in the cancer-affected area, as in images obtained with a transmission-type system. About 50% of the fluorescence emitted from the samples passed through the barrier filter and were detected by the ICCD camera. Theoretically, therefore, the input fluorescence is reduced by 25% compared with that of transmitted-type imaging systems. This reflected-type imaging system uses an ICCD camera of higher sensitivity than the one used in the previously reported transmitted-type imaging system.

However, even when ICG-sulfo-OSu and this imaging system are used, visualization of target lesions is still limited. It would be better if monoclonal antibodies with a higher tissue specificity and fluorescence reinforcing agents (13) could be developed. Also a major problem with this new method of endoscopic diagnosis is that it is still unknown whether immune reactions to these monoclonal antibodies actually take place in vivo. The mucous barrier (14) in the digestive tract may suppress immune reactions between the lesion and the labeled...
antibody. No previous study has been able to demonstrate the binding of labeled antibodies (administered into the digestive lumen) to the lesions at the tissue level.

In this study, 5 cases of colon cancer were investigated. IR fluorescent image was performed in all patients. Satisfactory fluorescent images were obtained regardless of their histological types. Concerning the immune response for colon cancer, the proportion of ICG-sulfo-OSu labeled anti-CEA antibody-positive cases depends on the cross-reactivity of native anti-CEA antibody. The sensitivity of native anti-CEA antibody to colon cancer was not 100%. Therefore, detailed data on the positivity of ICG-sulfo-OSu labeled anti-CEA antibody for colon cancer should be further examined.

Although, localization was similar between the sites stained with DAB-positive site and the IR fluorescent image, these sites were not completely consistent. This may have been due to the following reasons: (a) although antibodies responded, the labeling substance, ICG-sulfo-OSu, had leaked from the site, and (b) although antibodies responded, the fluorescence was weak due to the low concentration of antibodies. These issues should also be further investigated.

These and other issues need to be resolved in order to move toward the goal of establishing a technique of immunostaining in vivo. We believe, however, that this procedure, i.e., the vital immunofluorescence method of minute cancer detection under infrared ray excitation, may be realized with ICG-sulfo-OSu-labeled antibody. Medical technology, particularly in the fields of molecular biology and electronic medical equipment, continues to progress rapidly. In the near future, we expect that new types of endoscopes, which can detect cancer-specific antibodies or very small amounts of fluorescence, will be used to establish diagnostics of minute cancer of the digestive tract could not distinguish from benign lesion by ordinary videendoscopy.

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References