A Novel Method for Detecting Single Glandular Intestinal Metaplasia in the Mucosal Surface of the Fixed Stomach Using Methylene Blue

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A close association between intestinal metaplasia of the stomach and the well-differentiated type of gastric cancer is well recognized. The etiological relationship and how intestinal metaplasia contributes to gastric carcinogenesis are, however, still unclear. In order to answer this question, precise mapping and identification of the smallest lesion of intestinal metaplasia are desired. Establishment of an accurate and easy method for detecting intestinal metaplasia was the goal of this study. Surgical specimens of stomachs resected for gastric cancer were used. The specimens were stained with methylene blue, an oxidation-reduction marker, in whole mount, after fixation with 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4), and observed under a stereomicroscope. Normal gastric mucosa was stained blue, whereas intestinal metaplasia mucosa was not stained and had white or sky-blue island-like features. Intestinal metaplasia of complete type was unstained and showed white island-like features, while intestinal metaplasia of incomplete type showed sky-blue staining. With this method, we were able to detect even intestinal metaplasia composed of a single gland, when the intestinal metaplasia was of complete type. When stomach samples were stained in the presence of diphenyleneiodonium chloride (DPI), an inhibitor of nicotineamide adenine dinucleotide phosphate reduced form (NADPH) reductase, all the samples were homogeneously stained blue. Loss of the color of methylene blue was caused by the reductase activity of NADPH reductase, which is strongly and specifically expressed in intestinal metaplasia. A novel method for detecting intestinal metaplasia, even a single gland, was established.

Key words: Intestinal metaplasia — Methylene blue — NADPH diaphorase — Cytochrome P-450 reductase — Gastric cancer

Intestinal metaplasia in the stomach is one of the most commonest forms of metaplasia in humans.[1] An association between intestinal metaplasia and intestinal-type gastric cancer has been reported both epidemiologically and histologically.[2–7] There are three possible explanations for this[2, 3] :(1) intestinal metaplasia is a direct precursor lesion of the cancer; (2) intestinal metaplasia creates a favorable milieu for carcinogenesis, perhaps by raising the pH of gastric juice, and improving the growth conditions for bacteria that produce carcinogens; or (3) intestinal metaplasia may simply be a paraneoplastic lesion caused by the same agents that gave rise to the cancer.[2] We previously reported that almost all intestinal metaplastic mucosa detected by the Tes-tape method,[8] in samples of ϕ3 mm in diameter, contains intestinal metaplastic glands of polyclonal origin.[9] This finding favors the hypothesis that intestinal metaplasia is not a monoclonally expanding direct precursor of intestinal-type gastric cancer.

Intestinal metaplasia can be classifed histologically into two or three types: a complete type (Type I) and an incomplete type (Type II).[4–7, 10, 11] The latter is classified into 2 subtypes (Type IIa and Type IIb) in some reports. Subtype IIb intestinal metaplasia, which is a variety of the incomplete type and is associated with sulfomucins, in particular, is known to accompany the intestinal type of gastric cancer.[4–7, 10–12]

Intestinal metaplasia glands are reported to arise initially in the proliferating zone at the neck of normal glands.[14] Once cells of the intestinal type arise, they replace normal glandular cell types throughout the gland.[13] We previously reported that about half of intestinal metaplasia glands are polyclonal in origin even though they arise in the antrum, which is originally covered with pyloric glands that are 95% monoclonal.[9] However, a polyclonal cell population cannot arise from a monoclonal cell population without contributions from other cell lineages. There must be ways for intestinal metaplasia glands to develop other than those that have been reported. A method of detecting very early intestinal metaplasia lesions is needed to analyze the process by which intestinal metaplasia arises.

Herein, we report a new method of detecting intestinal metaplastic lesions on the mucosal surface of the fixed stomach. This new method allows detection of intestinal metaplasia even when comprising only a single gland. The

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method is based upon specific expression of NADPH diaphorase activity. In the well-known detection method for intestinal metaplasia using methylene blue under endoscopy, the intestinal metaplasia is stained blue, due to the absorptive function of intestinal metaplasia. The method reported herein is completely different, because the intestinal metaplasia is not stained.

MATERIALS AND METHODS

Specimens Sixteen gastrectomy specimens were obtained from 16 gastric cancer patients who were operated on at the National Cancer Center Hospital East, Japan, between October 1995 and March 1998.

Detection of intestinal metaplasia with methylene blue
The specimens were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) at 4°C for 3–4 h. They were then washed in phosphate-buffered saline (PBS) (pH 7.4) and stained with 0.2% methylene blue in 0.1 M PB (pH 7.4) for 2 min. After again being washed in PBS, they were examined under a stereomicroscope and detected lesions were photographed.

Details are presented in "Results." White lesions (white islands) and sky-blue lesions (sky-blue islands) were detected under a stereomicroscope after staining with methylene blue. The photographs were analyzed using a software package (NIH Image 1.61) and particle densities of the 70 white islands and the 50 sky-blue islands were compared in terms of the density of the stained area around the islands.

Histological analysis Gastric mucosa containing the lesions detected was sampled, by punch biopsy, to obtain 3-mm-diameter or 6-mm-diameter specimens with a Dispo-punch (Maruho Co., Osaka). Seventy samples of mucosa containing a white lesion (white island) even after staining with methylene blue, 50 samples of mucosa containing a sky-blue lesion (sky-blue island), and 50 samples of mucosa completely stained with methylene blue were sampled. The mucosal samples were fixed with 10% formaldehyde overnight, then dehydrated, penetrated, and embedded in paraffin. Mucosa samples containing minute lesions, as well as completely stained samples, were cut into 4-μm serial sections parallel to the mucosal surface, and the other mucosa samples were cut into 4-μm serial sections perpendicular to the mucosal surface. They were then deparaffinized, and some were stained with hematoxylin and eosin and others were stained with high iron diamine (HID)-Alcian blue (pH 2.5). The stained sections were analyzed by light microscopy.

Prevention of detection with diphenyleneiodonium chloride (DPI) We previously found that intestinal metaplasia exhibits nicotineamide adenine dinucleotide phosphate reduced form (NADPH) oxidoreductase activity while normal gastric mucosa does not.[15] DPI (Sigma-Aldrich,

Tokyo) is an inhibitor of NADPH oxidoreductase activity,[16] and we have already confirmed that DPI inhibits NADPH oxido-reductase staining on cryosections.[15] Methylene blue is an oxidation-reduction marker that appears blue in the oxidized state and colorless in the reduced state. We investigated whether detection of the islands would also be prevented by DPI.

A piece of gastric mucosa was divided into two mirror halves after fixation with 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 3–4 h at 4°C. One of the two pieces was treated with DPI and the other was not. A stock solution of DPI was prepared at a concentration of 10 mM in dimethylsulfoxide (DMSO), and was diluted to 25 μM in 0.1 M PBS or methylene blue solution prior to use. All of the procedures for detection of intestinal metaplasia were performed as described above, except for the use of 25 μM DPI in PBS and methylene blue solution. In this experiment, PBS and methylene blue solution containing 0.2% DMSO without DPI were used for the control specimens.

NADPH oxidoreductase staining Five white islands and 5 sky-blue islands were sampled from the gastric mucosa. They were sliced and stained by a method reported previously.[15] Briefly, they were embedded in OCT compound (Sakura, Tokyo) and cut into 10-μm sections with a cryostat. The sections were blocked with 0.1% Triton X-100 in 0.1 M PBS overnight at 4°C and then incubated in 0.1 M PBS containing 0.1% Triton X-100, 0.01% nitroblue tetrazolium, and 0.05% β-NADPH for 20–60 min at 37°C. The reaction was stopped by washing with 0.1 M PBS.

RESULTS

Detection of intestinal metaplasia with methylene blue
The gastric mucosa was examined under a stereomicroscope after staining with methylene blue. There were regions that had not stained (white islands) and others that stained only slightly (sky-blue islands). A representative mucosal specimen in which both a white island and sky-blue islands are seen in the same view is shown in Fig. 1A. The white islands were very clear. The sky-blue islands were vague, compared to the white islands, but a pit pattern like that in and around the stained mucosa was undetectable in the islands and could be distinguished from that around the region. Some mucosal regions had no islands at all, and others had either islands or larger fields, which may have been formed by fusion of adjacent islands.

The islands were sampled and analyzed histologically. The gross appearance of representative islands and their histological appearance are shown in Fig. 1, B–D. All regions that contained islands showed intestinal metaplasia, and the size of the intestinal metaplasia lesions on the sections correlated with island size. Intestinal metaplasia of the white islands contained Paneth's cells and was

referred to as the complete type of intestinal metaplasia or intestinal metaplasia type I.[8] Intestinal metaplasia of the sky-blue islands did not contain Paneth's cells, but sulfomucin was sometimes present. This is the incomplete type of intestinal metaplasia, or intestinal metaplasia subtype IIa and subtype IIb.[2–7, 10–12]

Fifty regions that did not contain islands were also examined histologically. Step sections were made parallel to the mucosal surface, but no intestinal metaplasia was detected in these regions.

One mucosal region contained a very small white island that showed a single gland of complete-type (Type I) intestinal metaplasia. The island and its histological appearance are shown in Fig. 2, A–D. It branched in about the middle portion of the gland, but the upper part had a single lumen, and appeared to be a single intestinal metaplastic gland. Small islands detected by this method, like this single gland, were usually surrounded by infiltration of inflammatory cells.

NADPH oxidoreductase staining Methylene blue is used as an oxidation-reduction indicator. It is blue in oxidized states and becomes colorless in reduced states. We previously found that gastric intestinal metaplasia contains NADPH oxidoreductase activity and that normal gastric mucosa does not.[15] We therefore hypothesized that white and sky-blue islands were detected by methylene blue because of this oxidoreductase activity.

Five regions containing white islands and five regions containing sky-blue islands were sampled for staining. Representative results are shown in Fig. 3. Intestinal metaplasia in white islands showed stronger staining, and intestinal metaplasia in sky-blue islands showed weaker staining. Gastric glands on the same sections that did not histologically exhibit intestinal metaplasia were unstained.
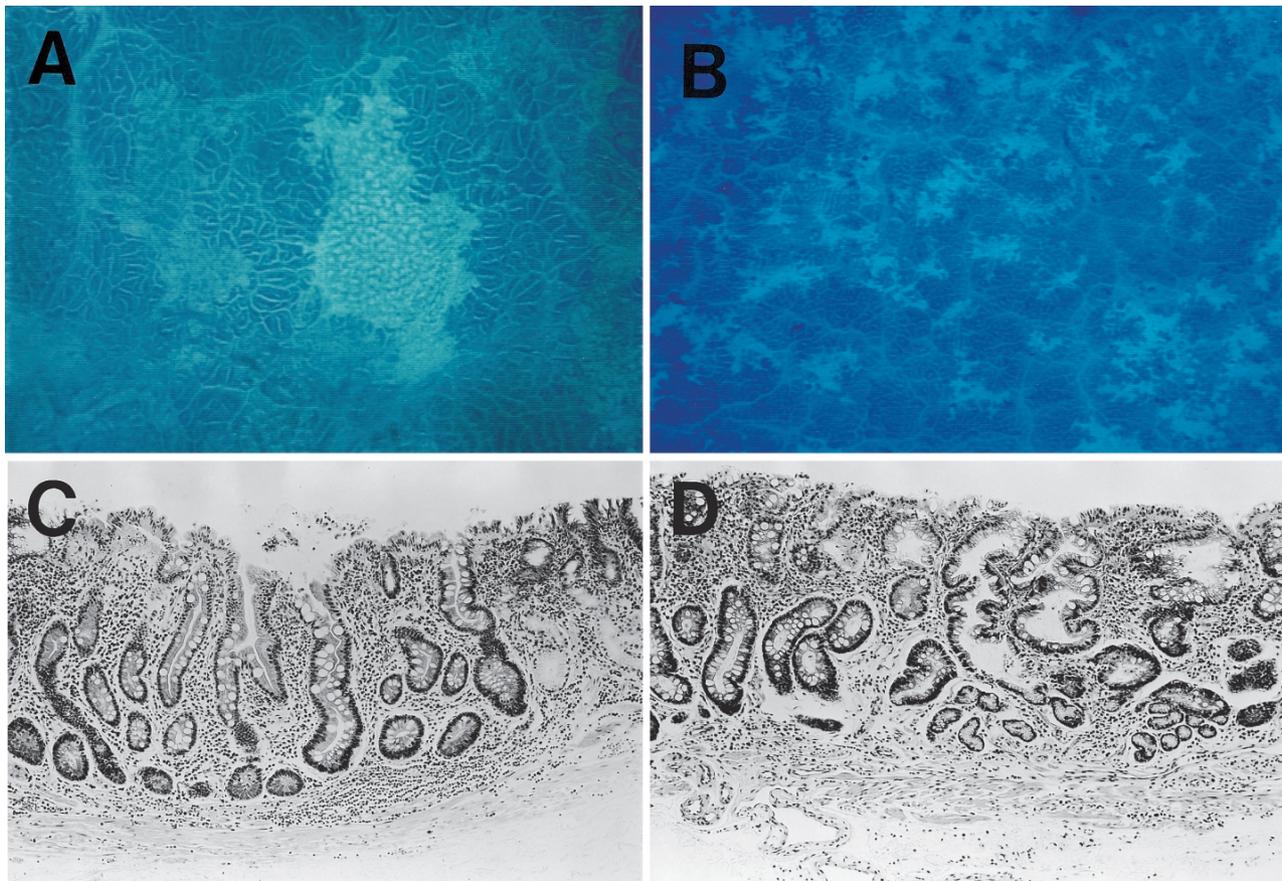
Fig. 1. A. Representative gastric mucosa after methylene blue staining. A single white island and several sky-blue islands are seen in a blue field. (×20, methylene blue, stereomicroscope). B. Low magnification view of gastric mucosa after methylene blue staining. There are many white islands in the blue field. (×9, methylene blue, stereomicroscope). C. Histological section of gastric tissue from a white island showing intestinal metaplasia with Paneth's cells. This form of intestinal metaplasia is the complete type or Type I. (×100, hematoxylin and eosin). D. Histological section of gastric tissue from a sky-blue island showing intestinal metaplasia without Paneth's cells. This intestinal metaplasia is the incomplete type or Type II. (×100, hematoxylin and eosin).
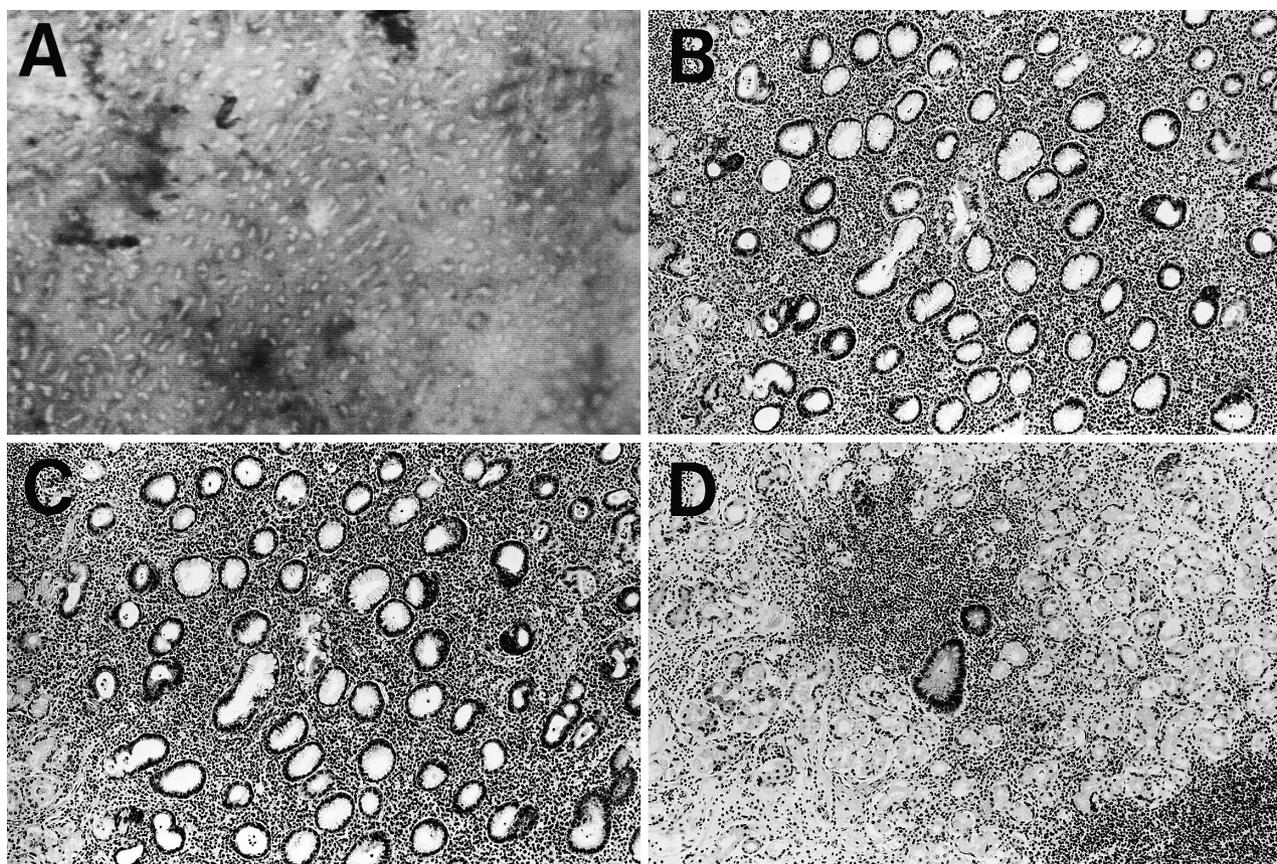
Fig. 2. A. A very small white island on the gastric mucosa after methylene blue staining. (×70, methylene blue, stereomicroscope). B–D. Cross sections of the mucosa in A. B shows the upper part, C shows the middle part, and D shows the lower part of the mucosa. There is a single intestinal metaplastic gland among normal gastric glands. The gland branches into 2 between the middle to lower level. (×100, hematoxylin and eosin).
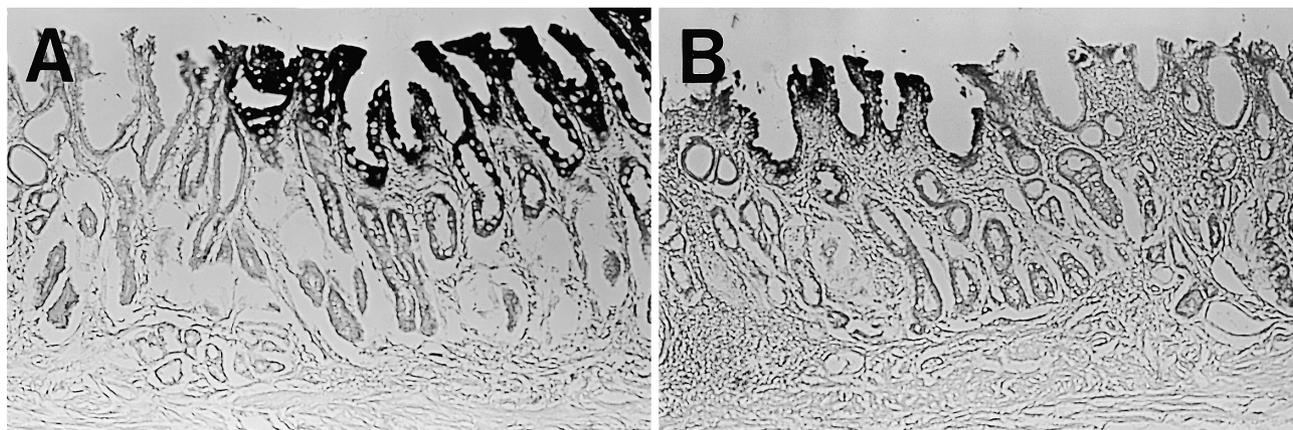
Fig. 3. A. An oxidoreductase stained section of gastric mucosa from a white island. It shows strong violet staining. (×100). B. An oxidoreductase stained section of gastric mucosa from a sky-blue island. It shows weaker violet staining. (×100).

DPI prevented staining Several white and sky-blue islands were detected on the piece not exposed to DPI, but none were detected on the piece treated with DPI. Even large islands on the mucosa not untreated with DPI at the edge of the separation did not continue on the other piece of mucosa treated with DPI (Fig. 4), but intestinal metaplasia was histologically observed to extend continuously beyond the gap in the mucosa.

DISCUSSION

The Tes-tape method has long been used to detect intestinal metaplasia in surgical specimens and it is a good method for use on fresh (not fixed) stomach specimens.[8] Intestinal metaplasia composed of a small number of glands, however, cannot be detected by the Tes-tape method, because the glucose produced spreads on the Tes-tape, and this method is based on imprinting enzyme activity onto the Tes-tape. Our new method using methylene blue can detect even a single gland of intestinal metaplasia. We stained 35 gastric specimens with gastric cancer of differentiated type by this method and found that all of them have intestinal metaplasia near the cancer. But intestinal metaplasia of 8 specimens among them could not be detected by the usual histological analysis. One of the shortcomings of this method is that it does not work without fixation. Washing the mucosa with saturated NaCl solution enhanced detection by this method without fixation, probably by removing mucinous material covering the mucosa, but intestinal metaplasia was not detected as clearly as in fixed mucosa. It would be impossible to detect single glandular intestinal metaplasia by washing with saturated NaCl solution. When unfixed intestinal metaplastic tissue is required to be used, the Tes-tape method would be better.[8]

We found previously that intestinal metaplasia contains NADPH oxidoreductase activity attributable to cytochrome P450 reductase[15] and hypothesized that the methylene blue method might be based on this enzymatic activity, because it is an oxidation-reduction marker. DPI, an inhibitor of NADPH oxidoreductase activity, prevented detection of intestinal metaplasia with methylene blue.[16] The complete type of intestinal metaplasia showed white islands and strong NADPH oxidoreductase activity histochemically, whereas the incomplete type showed sky-blue islands and weak activity. In view of the above, this new method of detecting intestinal metaplasia appears to be based on the NADPH oxidoreductase activity of cytochrome P450 reductase in intestinal metaplasia. However, leukomethylene blue is reported to be autoxidizable in air. In the case of methylene blue, reoxidation, either enzymatic or non-enzymatic, seems to be suppressed. We did not identify the suppression mechanism in this study. This suppression is not due to either paraformaldehyde or formaldehyde, because even saturated NaCl solution allowed detection.

This method is very easy to perform. It takes a total of about 4 h, including 3 h for fixation. Although mainly 4% paraformaldehyde in 0.1 M PB (pH 7.4) was used for fixation in this study, buffered 10% formaldehyde was also tried. The islands can be detected after fixation with buffered 10% formaldehyde for 2 h. They can be detected even after 48 h, but the color contrast between the intestinal metaplastic mucosa and the normal mucosa dimin-
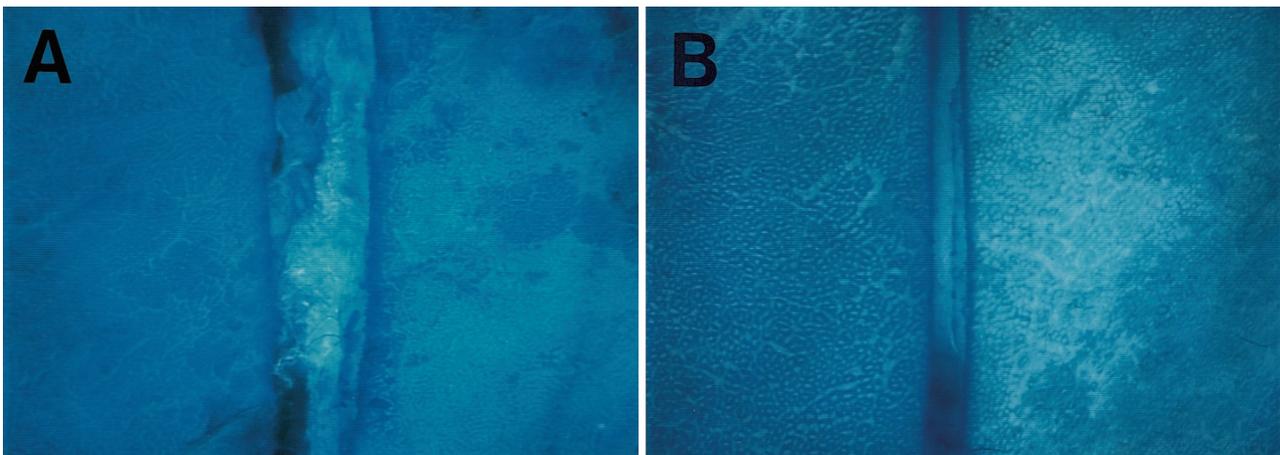
Fig. 4. A, B. Continuous gastric mucosa stained with methylene blue after having been divided in two. The right side was stained without DPI and the left was stained with DPI. A. A large white island is seen on the right, but the rest of the island is undetectable on the left. (×7, methylene blue, stereomicroscope). B. A large sky-blue island is seen on the right, but the rest of the island cannot be detected on the left. (×7, methylene blue, stereomicroscope).

ishes. NADPH oxidoreductase is reported to be resistant to degradation, with its activity being well preserved after 48 h of buffered formaldehyde fixation,[17] but it is gradually denatured by formaldehyde. The fixation time needed to assure good methylene blue staining was 2 h for 10% formaldehyde and within 24 h for 4% paraformaldehyde in 0.1 M PB (pH 7.4), to assure preservation of NADPH oxidoreductase activity.

The complete type and incomplete type of intestinal metaplasia were differentiated by the present method, but subtypes IIa and IIb could not be distinguished. We were unable to find a single gland of the incomplete type (Types IIa and IIb). The reason may be the sensitivity of this method. The white islands were easier to detect than the sky-blue islands in blue fields. There is a possibility of not being able to find all incomplete-type intestinal metaplasia by this method. We did not, however, find any areas of intestinal metaplasia in 50 blue regions without islands. It may be that the incomplete-type intestinal metaplasia initially arises in a group of glands, rather than in a single gland.

Intestinal metaplasia of the stomach has been shown to coexist frequently with the well-differentiated type of gastric cancer, but the role of intestinal metaplasia in the etiology of this cancer is still unclear.[2–7, 10–12] Cytochrome P450 enzymes are reported to bioactivate and to detoxify certain carcinogens and anticancer drugs.[18] It was hypothesized that P450 enzymes contribute to tumor promotion as well as initiation. The results of this study raise the possibility that intestinal metaplastic glands express cytochrome P450 enzymes, and this has recently been confirmed (Tatemichi *et al.*, unpublished results). These P450 enzymes are potentially major determinants of gastric

carcinogenesis, since P450 enzymes can activate chemicals in proximity to the targets. Small intestinal mucosa, which was stained with methylene blue as a control, was not stained blue and become blue after treatment with DPI, like intestinal metaplasia. As few cancers are found in small intestine, intestinal metaplasia itself will not be induced to develop into cancer by metabolites generated by P450 enzymes. But intestinal metaplasia may be creating a more favorable milieu for cancer generation in the surrounding gastric mucosa by activating carcinogens. Our results show complete-type intestinal metaplasia to have stronger P450 reductase activity than the incomplete type. This is inconsistent with a report that intestinal metaplasia of the incomplete type is found more frequently around cancers than the complete type. However, the reason for this discrepancy has not yet been clarified, and research aimed at resolving this issue is ongoing.

Our novel method may provide new clues in regard to the histogenesis of intestinal metaplasia and gastric cancer near intestinal metaplasia. Identifying very small intestinal metaplasia would help in finding very small cancers, and may open up a new field of research on intestinal metaplasia in relation to gastric carcinogenesis.

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