Successful outcomes of a novel endoscopic treatment for GI tumors: endoscopic submucosal dissection with a mixture of high-molecular-weight hyaluronic acid, glycerin, and sugar

Mitsuhiro Fujishiro, MD, PhD, Naohisa Yahagi, MD, PhD, Masanori Nakamura, MD, Naomi Kakushima, MD, Shinya Kodashima, MD, Satoshi Ono, MD, Katsuya Kobayashi, MD, Takuhei Hashimoto, MD, Nobutake Yamamichi, MD, PhD, Ayako Tateishi, MD, Yasuhito Shimizu, MD, PhD, Masashi Oka, MD, PhD, Keiji Ogura, MD, PhD, Takao Kawabe, MD, PhD, Masao Ichinose, MD, PhD, Masao Omata, MD, PhD

Tokyo, Japan

Background: Endoscopic submucosal dissection (ESD) has recently been developed for endoscopic treatment of GI tumors, which enables us to resect even large tumors en bloc. However, a considerable frequency of perforation has become another problem. The best way to prevent perforation is to create a sufficient submucosal fluid cushion (SFC). The aim of this study is to find out the feasibility of ESD by using a mixture of 1900 KDa hyaluronic acid (Suvenyl) and a 10% glycerin plus 5% fructose solution (Glyceol).

Methods: Sixty-seven consecutive GI tumors in 54 patients who met indication criteria of ESD were enrolled. The mixing ratios of Suvenyl and Glyceol were 1:3 for esophageal/colorectal tumors and stomach tumors with scar, and 1:7 for stomach tumors without scar. After creation of SFCs, mucosal incision around the tumors and submucosal dissection under the tumors were made by cutting devices. The clinical outcomes were investigated.

Results: Mean resected and tumor sizes were 38.6 and 25.6 mm, respectively. Perforation occurred in one colon tumor with severe fibrosis (1.5%), which was managed by endoscopic clipping without salvage surgery. No blood transfusion was performed. In one stomach and in one rectal tumor (3%), endoscopic hemostasis was necessary because of postoperative bleeding. Overall endoscopic and histologic en bloc resection rates were 94% (63/67) and 78% (52/67), respectively, and there was no recurrence after follow-up of 1 year.

Conclusions: ESD when using a mixture of Suvenyl and Glyceol results in excellent outcomes, and this injection solution should be used for ESD. (Gastrointest Endosc 2006;63:243-9.)

Tumors without lymph-node metastasis in the GI tract can be treated, theoretically, by intraluminal endoscopic treatments, e.g., EMR.[1,2] However, the indications of EMR had been limited to small mucosal tumors until recently because of technical limitations in the resected size (less than 2 cm in size) and then many mucosal tumors had been resected by surgery even though the possibility of lymph-node metastasis was extremely low. The endoscopic submucosal dissection (ESD) technique is a new endoscopic treatment that uses cutting devices, which remove the tumors by following 3 steps: (1) injecting fluid into the submucosa to elevate the lesion from the muscle layer, (2) pre-cutting the surrounding mucosa of the lesion, and (3) dissecting the connective tissue of the submucosa beneath the lesion.[3-8] The major advantages of the technique in comparison with conventional EMR are the following: first, the resected size and shape can be controlled; second, en bloc resection is possible even in a large tumor; and third, the tumors with ulcerative findings also are resectable. Some investigators[7,8] consider that the technique is independent from conventional EMR, because the potential outcomes are extremely different. Although the curability of the tumors by ESD is much higher than conventional EMR, the cut and the dissection steps that use cutting devices may be technically difficult, with a substantial risk of perforation. The best way to prevent perforation is to lift up the lesion sufficiently from the muscle layer. We previously reported that, in the animal models, a submucosal fluid cushion (SFC) created by a hyaluronic acid (HA) solution persisted for longer periods of time than other available submucosal injection solutions and

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a mixture of a high-molecular-weight HA solution and a 10% glycerin with 5% fructose plus 0.9% saline solution (Glyceol; Chugai Pharmaceutical Co, Tokyo, Japan) may be the best injection solution in terms of thickness of SFCs and tissue damage caused by injection solutions.[9-11] Our study evaluates the clinical outcomes of ESD that uses the best submucosal injection solution, based on the results of the animal studies.

Capsule Summary

What is already known on this topic

- ESD allows for en bloc resection of GI tumors.
- The creation of a sufficient submucosal cushion may prevent ESD-induced perforation.

What this study adds to our knowledge

- The mixture of high-molecular-weight hyaluronic acid, glycerin, and sugar creates a long-lasting submucosal cushion for a safer ESD.

PATIENTS AND METHODS

From November 2003 to March 2004, 67 GI tumors (10 esophagus, 26 stomach, 1 duodenum, 30 colorectum) in 54 consecutive patients, who gave written informed consent for participation in the study, were enrolled. The patients with tumors that met the following indication criteria of ESD at the University of Tokyo Hospital, Tokyo, Japan, were eligible for this study.

The tumors were diagnosed before surgery as the following:

- Esophagus: severe dysplasia, carcinoma in situ, intramucosal cancer
- Stomach: adenoma with severe atypia, intramucosal cancer with differentiated type and without ulcer findings; intramucosal cancer with differentiated type, less than 3 cm in size, and ulcer findings
- Duodenum: adenoma with severe atypia, intramucosal cancer with differentiated type
- Colorectum: adenoma with severe atypia, intramucosal cancer with differentiated type
- All the organs: small carcinoid tumors with less than 1 cm in size and without invasion into the muscle layer

All of these were tumors with technical difficulty to achieve en bloc resection by conventional EMR or snare polypectomy because of location, shape, size, or submucosal fibrosis from peptic ulcer or previous treatments.

Preparation of the submucosal injection solution

Submucosal injection solution used in this study was a mixture of a 1% 1900 KDa HA preparation (Suvenyl; Chugai) and Glyceol, with a small amount of indigo carmine and epinephrine. Indigo carmine was added to clarify the area of submucosal injection and to distinguish clearly between the muscle layer and the submucosal layer. Epinephrine was added to produce a higher hemostatic ability from contraction of small blood vessels. Indigo carmine (1 mL of 1%) and epinephrine (1 mL of 0.1%) were mixed in a 200-mL container of Glyceol, and 7.5 mL of the solution was drawn into a 10-mL disposable syringe to use for the esophageal/colorectal tumors and for the stomach tumors with ulcer findings. For the stomach tumors without ulcer findings, 17.5 mL of the solution in a 20-mL disposable syringe was used. The syringe that contained the mixed Glyceol solution and a syringe that contained 2.5 mL of Suvenyl were connected to a tripodal adaptor, and push and pull movements of the back of syringes were repeated approximately 10 times to mix both solutions. After mixing sufficiently, the mixture of Glyceol and Suvenyl was divided into 5-mL syringes to be ready to connect to a 23-gauge endoscopic injection needle (Varixor [23G/Type S]; Top Co, Tokyo, Japan).

ESD procedure

The tumors were treated by the standard ESD procedures by using the Flex knife (KD-630L; Olympus Optical Co, Tokyo, Japan) alone or in combination with the Hook knife (KD-620LR; Olympus). The equipment was a single-channel endoscope with water-jet system (XGIF-Q240M, Olympus; or EG-2931, Pentax Co, Tokyo, Japan), even in the colorectum and a high-frequency generator with an automatically controlled system (Endocut mode) (Erbotom ICC 200; ERBE Elektromedizin GmbH, Tübingen, Germany).

The ESD procedure is as follows (Fig. 1):

1. Marking dots were made by using the Flex knife with the soft coagulation mode (50 W for esophageal and stomach tumors) on the circumference of the target lesion. Marking dots were not made for duodenal and colorectal tumors because the margin of the tumors could be visualized clearly even after submucosal injection, and marking without submucosal injection in the thin wall may cause perforation.
2. Several milliliters of the above solution were injected, with a 23-gauge disposal injection needle, into the submucosal layer around the lesion to lift it off the muscle layer.
3. Incision of the mucosa outside the marking dots was made with the Flex knife with the Endocut mode (effect 3, 80 W for the stomach tumors; effect 2, 60 W for the esophageal, the duodenal, and the colorectal tumors) to separate the lesion from the surrounding nonneoplastic mucosa.
4. Injection of several milliliters of the above solution was given into the submucosal layer just beneath the lesion.
5. The submucosal connective tissue just beneath the lesion was dissected from the muscle layer by using the Flex knife with the forced coagulation mode (40 W for

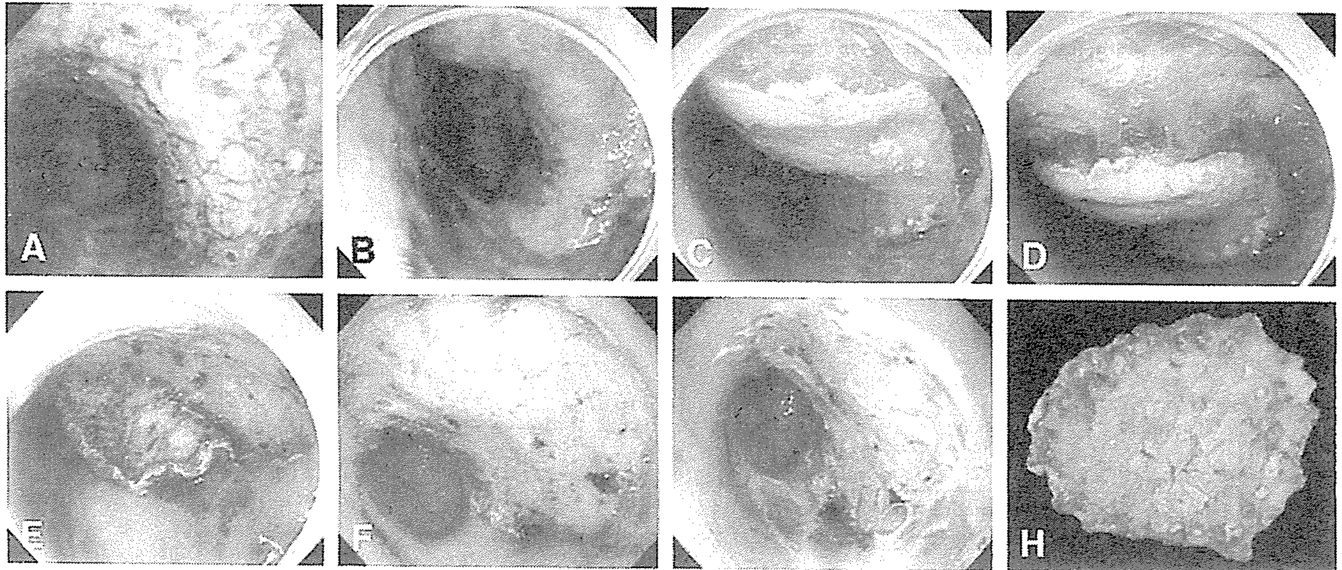

Figure 1. Endoscopic submucosal dissection. **A,** Marking dots are made on the circumference of an early type IIa gastric cancer. **B,** Several milliliters of the submucosal injection solution are injected around the lesion to lift it off the muscle layer. **C,** Incision of the mucosa outside the marking dots is made to separate the lesion from the surrounding nonneoplastic mucosa. **D,** The submucosal connective tissue just beneath the lesion is gradually dissected from the muscle layer. **E,** Half of the dissection is completed. **F,** The lesion is cut completely from the muscle layer. **G,** Sucralfate is sprayed on the artificial ulcer to coat its surface and to confirm hemostasis. **H,** The resected size is 65 × 58 mm, the lesion size is 44 × 42 mm, and the histologic en bloc resection is completed.

all tumors). Where it was difficult to dissect with the Flex knife, the Hook knife was used. For a large tumor, steps of 2 to 5 are repeated after an incision of a few centimeters of the mucosa and dissection of the submucosal connective tissue to keep lifting enough from the muscle layer before the submucosal injection solution poured out from under the lesion.

6. The raised lesion is removed with a standard polypectomy method with a snare (normal type) (SD-5L-1; Olympus) by using the Endocut mode (effect 3, 80 W for the stomach tumors; effect 2, 60 W for the esophageal, the duodenal, the colorectal tumors). Or, the raised lesion is cut off completely from the muscle layer without snaring.
7. Visible exposed vessels on the artificial ulcer were coagulated with hemostatic forceps (SDB2422; Pentax) or Coagrasper (FD-410LR; Olympus) with the soft coagulation mode (50 W for all tumors)
8. Sucralfate (Ulcermin; Chugai) was sprayed onto the artificial ulcer to coat its surface and to confirm hemostasis.[12]
9. Finally, the resected specimen was retrieved from the GI tract with a grasping forceps for histologic evaluation.
10. Patients without complications were permitted to take soft food a day after ESD and were discharged within 10 days. For esophageal, stomach, and duodenal tumors, a proton pump inhibitor (10 mg rabeprazole, 20 mg omeprazole, or 30 mg lansoprazole daily) and sucralfate (1 g, 3 times a day) were prescribed up to 2 months after ESD; no medication was prescribed for colorectal tumors.

Histologic evaluation

The resected specimens were fixed with formalin, cut into 2-mm slices, and embedded in paraffin. A histologic section was made from each block and was stained with H&E. Histologic assessment was microscopically performed in detail according to the Japanese Classification of Esophageal, Stomach, and Colorectal Carcinoma.[13-16] Because submucosal massive invasion, existence of undifferentiated-type cells, and/or vessel involvement after histologic evaluation were regarded as risks of cancer-positive lymph nodes, surgical intervention was strongly recommended.[17-19] Evaluation of the extension of tumor cells to the lateral margin was classified into the following 3 groups: complete resection, free of tumor glands on cut ends; incomplete resection, exposition of tumor glands on cut ends; and not evaluable, impossibility of evaluation because of a burn effect by diathermic treatment, mechanical damage, multipiece resection, etc.

Assessment of therapeutic efficacy: endoscopic and histologic en bloc resection

Endoscopic en bloc resection was defined when the tumor was resected in one piece and the rim of artificial ulcer after ESD was, endoscopically, free of tumor. Histologic en bloc resection was defined when the tumor was histologically resected in one piece, with complete resection as defined above. When ESD was completed, with resection of two or more specimens, it was not defined as endoscopic en bloc resection even in those with only one specimen, by using histology, that contained tumor glands. On the contrary, even in those treated with

TABLE 1. Clinopathologic features of the esophageal tumors*

Mean size, mm (range)		23 (7-60)
Location	Ce/Ut/Mt/Lt/Ae	0/3/3/3/1
Macroscopic type	IIa/IIb/IIc	1/1/8
Histologic depth	Dysplasia/adenoma	1
	Carcinoma in situ	3
	Mucosa (invasive)	5
	Submucosa	1
Vessel infiltration	Presence	0
	Absence	10

Ce, Cervical esophagus; *Ut*, upper thoracic esophagus; *Mt*, middle thoracic esophagus, *Lt*, lower thoracic esophagus; *Ae*, abdominal esophagus.
*Terminology is derived from Refs. 13, 16.

TABLE 2. Clinopathologic features of the stomach tumors*

Mean size, mm (range)		23 (2-70)
Location	U/M/L	4/11/11
Macroscopic type	IIa	10
	IIc	9
	IIc + IIa	3
	IIc with ulcer findings	2
	Recurrent tumor	2
Histologic depth	Dysplasia/adenoma	8
	Mucosa	14
	SM1	2
	SM2 or deeper	2
Vessel infiltration	Presence	1
	Absence	25

U, Upper third of the stomach; *M*, middle third of the stomach; *L*, lower third of the stomach; *SM*, submucosa.
Cut-off limit of depth of submucosal invasion between SM1 and SM2 is 500 μm in the stomach.
*Terminology is derived from Refs. 14, 16.

resection of two or more specimens, it was defined as histologic en bloc resection when the margin of the resected specimen that contained the whole tumor was free from tumor glands in a single piece and when the other resected specimens did not reveal any tumor glands by histology.

Complications: bleeding and perforation

Major bleeding was defined when massive bleeding during the procedure required blood transfusion or when postoperative bleeding required hemostatic treatment, such as endoscopic clipping, thermocoagulation, and/or injection therapy. To evaluate minor bleeding, changes of blood Hb levels between pre-ESD (a day before ESD) and post-ESD (a day after ESD) were measured. Perforation was diagnosed endoscopically when another abdominal organ, mesenteric fat, or intra-abdominal space was observed during the procedure and/or by the presence of free air in the peritoneal cavity, or air extending into the retroperitoneal or mediastinal space in the plain radiograph.

Recurrence after ESD

In case of esophageal, stomach, and duodenal tumors, follow-up endoscopies were principally performed within a week after ESD to check visible vessels before discharge, 2 months after ESD to confirm artificial ulcer healing, and 6 months and 1 year after ESD to look for recurrent tumors and secondary tumors. In case of colorectal tumors, follow-up endoscopies were principally performed 2 months after ESD to confirm artificial ulcer healing, and 6 months and 1 year after ESD to check for recurrent tumors and secondary tumors. To check for distant recurrence, patients with the cancerous lesions underwent chest and abdominal CTs for esophageal tumors, and abdominal and pelvic CTs for stomach and colorectal tumors 1 year after ESD by the decision of the doctors in charge.

RESULTS

Clinicopathologic features

Clinicopathologic features of the enrolled tumors are shown in Tables 1 to 3. One patient with a duodenal tumor who was enrolled in this study had a type IIa, 15-mm adenoma located on the posterior wall of the duodenal bulb, and the tumor was resected by the specimen of 22 mm in size. A mean resected size was 38.6 mm (esophagus, 35 mm; stomach, 47 mm; colorectum, 33 mm) and a mean tumor size was 25.6 mm (esophagus, 23 mm; stomach, 23 mm; colorectum, 29 mm). Even small tumors also were treated by ESD because of a difficult location by using conventional EMR or the existence of a scar. Eight tumors (4 stomach tumors and 4 colorectal tumors) had scars, seen on histologic examination, because of accompanying peptic ulcers, previous intensive biopsies, or previous endoscopic treatments. Among 67 tumors, 63 tumors (94%) (esophagus, 9; stomach, 24; duodenum, 1; colorectum, 29) were considered as node-negative tumors histologically and were followed without additional treatments. One esophageal tumor was diagnosed after ESD as a submucosal invasive tumor, and additional radiotherapy was performed. In the stomach tumors, two tumors were diagnosed after ESD as tumors with massive invasion into the submucosa with/without lymph-vessel infiltration. Both

tumors were treated by additional gastrectomy with lymph-node dissection, which resulted in no remnant tumors. In the colorectal tumors, one carcinoid tumor was diagnosed after ESD as a tumor with massive invasion into the submucosal layer and with vessel infiltration, although the size of the tumor was 5 mm in the greatest diameter. The case was closely followed, without surgical intervention because of the patient's desire. The other tumors of the stomach and the colorectum with minute invasion into the submucosa were closely observed without additional surgery, because recent studies revealed that lymph-node metastasis of such lesions was almost zero.[18,19]

En bloc resection rates

An overall endoscopic en bloc resection rate was 94% (63/67) (esophagus, 100%; stomach, 100%; duodenum, 100%; colorectum, 87%), and precise histologic assessment was performed in all the tumors. When considering histologic evaluation, an overall histologic en bloc resection rate was 78% (52/67) (esophagus, 80%; stomach, 92%; duodenum, 100%; colorectum, 63%) (Table 4).

Complications

Minor bleeding was encountered in all the tumors when incising the mucosa or when dissecting in the submucosal layer, but hemostasis was achieved with thermocoagulation or endoscopic clipping during the procedures. A mean change of blood Hb levels between pre- and post-ESD was −0.29 g/dL (range 2.1∼+0.8 g/dL). The Hb levels dropped by more than 1 g/dL in 5 of 54 patients (9.2%) (stomach, 3; colorectum, 2), and by more than 2 g/dL in only one patient with a stomach tumor (1.9%). In this case, the tumor was a 19-mm adenoma, which was located on the posterior wall of the gastric lower body. The procedure of ESD was completed in a short time without complications, and bleeding during ESD was minor. So, the bleeding might occur after ESD without any symptom, and spontaneous hemostasis might be achieved.

No patient had massive hemorrhage that needed blood transfusion. In two tumors (stomach, 1; rectum, 1), endoscopic hemostasis was performed because of postoperative bleeding. In case of the stomach tumor, bleeding within a day after the procedure was noticed by hematemesis. Emergency endoscopy revealed bleeding from the visible vessel on the artificial ulcer base, and hemostasis was obtained with endoscopic clipping. In the case of the rectal tumor, bleeding 7 days after the procedure was noticed by massive rectal bleeding, which also was controlled by endoscopic clipping.

Perforation was experienced in a patient with one colon recurrent tumor (1.5%), which had severe fibrosis in the submucosal layer because of a previous conventional multifragmental EMR. In this case, endoscopic clipping for disrupted muscle fibers prevented salvage surgery without any further complication. The patient had mild abdominal symptoms without fever for a few days and recovered well with 3-day fasting and antibiotics administration.

TABLE 3. Clinopathologic features of the colorectal tumors*

Mean size, mm (range)		29 (5-91)
Location	C/A/T/D/S/R	0/8/7/3/4/8
Macroscopic type	Is	3
	LST-G	15
	LST-NG	7
	LST-NG with scar	2
	Recurrent tumor	2
	Carcinoid	1
Histologic depth	Dysplasia/adenoma	13
	Mucosa	12
	SM1	4
	SM2 or deeper	1
Vessel infiltration	Presence	1
	Absence	29

C, Cecum; *A*, ascending colon; *T*, transverse colon; *D*, descending colon; *S*, sigmoid colon; *R*, rectum; *Is*, protruded tumor with sessile shape; *LST-G*, laterally spreading tumor with granular type; *LST-NG*, laterally spreading tumor with nongranular type; *SM*, submucosa. Cut-off limit of depth of submucosal invasion between SM1 and SM2 is 1000 μm in the colorectum.
*Terminology is derived from Refs. 15, 16.

Recurrence after ESD

All the patients with ESD were successfully followed, by the doctors in charge, for more than 12 months after ESD. Physical examinations and routine laboratory tests revealed no evidence of tumor recurrence in all the cases. Except for two patients with additional gastrectomy, 52 patients with 65 tumors, including one rectal carcinoid tumor with vessel infiltration and one esophageal tumor with additional radiotherapy, underwent endoscopies after 12 months of ESD; the endoscopies revealed no local recurrence in any of them. CTs 12 months after ESD were taken for 23 tumors (esophagus, 6; stomach, 12; colorectum, 5), including the cases of additional treatments, because of invasive esophageal cancers, stomach cancers with submucosal invasion, large size, or because of ulcer findings, colorectal cancers with submucosal invasion, or a carcinoid tumor. Short-term follow-up of 12 months revealed no local or distant recurrence in any of them (Table 5).

DISCUSSION

Developments of medical technologies and discoveries of novel medical knowledge enable us to choose a minimal

TABLE 4. Endoscopic en bloc resection rate and histologic margin of the resected specimens

	Esophagus, % (no.)	Stomach, % (no.)	Duodenum, % (no.)	Colorectum, % (no.)
Endoscopic en bloc resection	100 (10/10)	100 (26/26)	100 (1/1)	87 (26/30)
Histologic margin				
Complete	80 (8/10)	92 (24/26)	100 (1/1)	63 (19/30)
Incomplete	0 (0/10)	4 (1/26)	0 (0/1)	13 (4/30)
Not evaluable	20 (2/10)	4 (1/26)	0 (0/1)	23 (7/30)

TABLE 5. Recurrence after endoscopic submucosal dissection during 1-y follow-up

	Esophagus, % (no.)	Stomach, % (no.)	Duodenum, % (no.)	Colorectum, % (no.)
Local recurrence	0 (0/10)	0 (0/24)	0 (0/1)	0 (0/30)
Distant recurrence	0 (0/6)	0 (0/12)	—	0 (0/5)

invasive treatment for cancer patients without ruining curability of tumors, especially in the fields of the gastroenterology. One of the ultimately minimal invasive treatments for GI tumors is ESD; however, we have to take substantial risks into account, as well as expected benefits. ESD has not been widely performed yet, even in Japan, because the procedure is more complex and the risks accompanying the procedure are higher than conventional EMR. Many GI tumors that are considered to be mucosal and differentiated-type without lymph-node metastasis are treated by multifragmental EMR, which may result in an inappropriate histologic diagnosis and a high recurrent rate, or surgery, which also may result in postsurgical dysfunction. If the risks of ESD are lessened, ESD may come to a standard treatment for node-negative GI tumors. The efforts to lessen the risks of ESD have been performed vigorously, and one of them was an innovation of a novel submucosal injection solution. Among available submucosal injection solutions in clinical practice, we previously revealed that the most suitable one for producing and maintaining long-lasting SFCs was a high-molecular-weight HA solution.[10] Furthermore, Glyceol, containing glycerin and fructose, was the best mixing solution for HA instead of normal saline solution, because glycerin could produce hypertonic potency over extracellular fluid without tissue damage, and fructose could increase viscoelasticity of an HA solution, making cross-linking of HA molecules. The concentration of 0.125% for a 1900 KDa HA solution made by Glyceol used for stomach tumors without scar was determined by the ability to create a similar SFC with a 0.5% 800 KDa HA solution made by normal saline solution,[9] which comes into outstanding results of ESD in the stomach tumors.[4-6] In comparison with the stomach wall, the esophageal, duodenal, and colorectal walls are fairly thin; therefore, it is favorable to increase the concentration used for these organs, and, in this study, a doubled concentration, 0.25%, was applied in such organs. Furthermore, it is impossible to create sufficient SFCs in the tumors with scar, therefore, the concentration of 0.25% also was applied in the stomach tumors with scar.

In this study, endoscopic en bloc resection was achieved in all the tumors except for 4 colorectal tumors. The reasons of resection with two or 3 specimens in the 4 colorectal tumors were in a tumor location where it was difficult to manipulate the endoscope and fibrosis, which prevented a sufficient SFC in the submucosal layer. These cases were snared halfway through dissecting the submucosa to avoid an increasing risk of perforation by continuing submucosal dissection or to rescue after perforation. We have to stress that when considering the risks and the benefits, again, and, regardless of multifragmental resection, if they are resected in a few pieces, there is a high possibility that they can be histologically evaluated and treated, without recurrence. From a technical standpoint, the existence of tumors with multifragmental resection shows that ESD is still in a developmental stage and further refinements of ESD techniques may be necessary to replace the standard treatments.

In comparison with an extremely high rate of endoscopic en bloc resection, the rate of histologic en bloc resection (one-piece resection with histologic tumor-free margins) is considerably low. It is well understood that it

is important to confirm complete resection by histologic examination, but, on the other hand, we have to consider postoperative disorders, such as stenosis or increasing risks of complications that may occur after wide mucosal resection, especially in the organs with narrow and angulated lumens, such as the esophagus, the duodenum, and the colorectum. Therefore, the mucosal incision was made very close to the tumors, which resulted in histologic judgment of incomplete or not-evaluable resection. Another reason was that fragile nature of the mucosa made the resected specimens torn off in some parts during collecting and stretching them. The margins of the tumors, except for some stomach tumors, are clearly identified endoscopically, so it is rare to mistake the tumor margins. But, in the stomach, there are some possibilities that marking dots were misplaced because of endoscopic blurred margins. We have to be careful of local recurrence, especially in the stomach, when the histologic evaluation revealed incomplete or not-evaluable resection, although this study revealed recurrent-free results in all the tumors. Inversely, additional treatment has to be watched for until the evidence of local recurrence is obtained by follow-up endoscopy, because there is the possibility of complete resection, even when the histologic evaluation was different.

We experienced two cases of post-ESD hemorrhage and one case of perforation in this study. The numbers of complications are extremely lower than previous reports of ESD.[3,7,20] Almost all post-ESD hemorrhage can be preventable, if we treat all the visible vessels on the artificial ulcer base after tumor resection when using hemostatic forceps or a Coagrasper, as this study shows. With regard to perforation, sufficient SFCs produced by the submucosal injection solution may be very effective. The only one case of perforation with severe fibrosis in the submucosal layer shows the limitation of lesion lifting for the case of severe fibrosis even when using the powerful submucosal injection solution. To prevent perforation in such a case, we may have to consider another approach such as innovation of devices or accessories.

In summary, ESD by using a mixed solution of high-molecular-weight HA, glycerin, and sugar for the GI tumors gave far excellent results in comparison with previous studies, and the injection solution should be used, especially for the difficult cases of ESD. After taking a few more steps in technologies and knowledge, ESD may become a standard treatment of all GI tumors without lymph-node metastasis.

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Current affiliations: Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; Second Department of Internal Medicine, Wakayama Medical University, Wakayama, Japan.

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Reprint requests: Mitsuhiro Fujishiro, MD, Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, Japan.

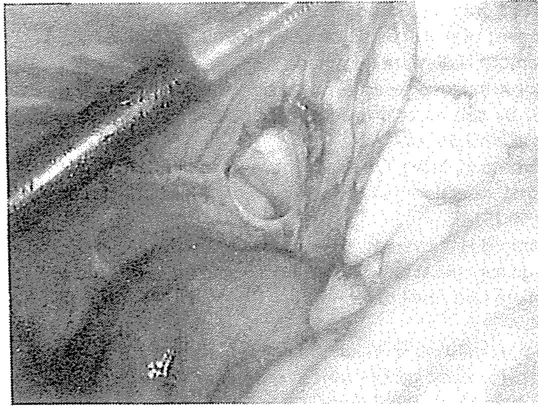

Figure 3. Hernial orifice.

preexisting hernia,[13] or herniation at a weakness of the abdominal wall from a scar.[14] A similar mechanism could be distension caused by whole-gut lavage, with an early onset after the preparation. The literature describes incarceration of a preexisting hernia.[16,17]

To our knowledge this is the first report of a Richter's hernia as a complication of colonoscopy. Distension of the bowel by air insufflation might be the mechanism responsible. This unusual presentation of a Richter's hernia illustrates the need to be aware of late complications of colonoscopy.

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Current affiliation: Departments of Surgery and Gastroenterology, Kantonsspital, Chur, Switzerland.

Poster presentation of the case at European Hernia Congress, March 29-April 2, 2004, Prague, Czech Republic.

Reprint requests: Peter Nussbaumer, MD, Department of Surgery, Kantonsspital Chur, Loestrasse 170, 7000 Chur, Switzerland.

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Successful endoscopic en bloc resection of a large laterally spreading tumor in the rectosigmoid junction by endoscopic submucosal dissection

Mitsuhiro Fujishiro, MD, Naohisa Yahagi, MD, Naomi Kakushima, MD, Shinya Kodashima, MD, Masao Ichinose, MD, Masao Omata, MD

Tokyo and Wakayama, Japan

In principle, a malignant or premalignant lesion ideally should be resected as a single piece, with adequate tumor-free margins to achieve a curative resection and to prevent tumor recurrence. For these reasons, surgery generally is recommended for large colorectal tumors. However, recently it has been recognized that large, flat-elevated colorectal tumors, so-called laterally spreading tumors (LST), tend to have a relatively benign course despite their large size, and, hence, endoscopic removal by using piecemeal EMR techniques has become widely accepted.[1]

Although large LSTs may be resected by using piecemeal EMR techniques, studies have shown high rates of recurrence after piecemeal resection.[1,2] In recent years, a new technique of endoscopic resection with cutting knives has been described for the en bloc resection of large and ulcerative lesions in the stomach.[3-5] This technique has been termed endoscopic submucosal dissection (ESD) by Japanese endoscopists as a distinct method from EMR, because the treatment outcomes are extremely different from other EMR techniques. ESD with an insulated-tip diathermic knife,[6] or a needle knife with sodium hyaluronate,[7-9] has been shown to be safe and effective for lesions in the rectum. We report here a case of a large LST located at the rectosigmoid junction, resected by ESD by using a combination of a flex knife and a submucosal injection of hyaluronic acid solution.

CASE REPORT

A 28-year-old gentleman with no significant medical history underwent a total colonoscopy for symptoms of rectal bleeding. This revealed a large, granular-type LST, over 9 cm in diameter, at the rectosigmoid junction (Fig. 1). The patient was subsequently referred to our hospital for treatment of the large LST. Detailed examination with chromomagnification endoscopy and EUS confirmed that the lesion was an intramucosal tumor. After discussion of the possible options of surgery, piecemeal EMR, ablation therapy, as well as ESD, written informed consent was obtained for performing ESD. The procedure was carried out without sedation by using a single-channel upper-GI endoscope with water-jet system (EG-2931; Pentax Co, Tokyo, Japan) and a high-frequency automated electrosurgical generator (Endocut mode) (Erbotom ICC 200; ERBE Elektromedizin GmbH, Tübingen, Germany). A transparent attachment (D-201-11804; Olympus Optical Co, Ltd, Tokyo, Japan) was fitted onto the tip of the endoscope, both to maintain endoscopic visualization and also to retract the connective tissue of the submucosal layer, to facilitate dissection.

The ESD procedure

A schematic of the procedure is shown in Figure 2.

1. Creating a submucosal fluid cushion. A mixture of 1% (1900 kDa) hyaluronic acid preparation (Suvenyl; Chugai Pharmaceutical Co, Tokyo, Japan), 10% glycerin, and 5% fructose plus 0.9% saline solution (Glyceol; Chugai) with a small amount of added epinephrine (1:200,000), and indigo carmine (1:20,000) was used as the submucosal injection solution. The two solutions were premixed in a ratio of 1:3. By using a 23-gauge injection (sclerotherapy) needle, repeated injections of approximately 2 mL of solution were used to raise the lesion and the surrounding mucosa.

2. Incising the mucosa outside the lesion. After lifting the mucosa, a circumferential incision was made around the lesion with the flex knife (KD-630L; Olympus) (Fig. 3). The flex knife was fixed at a length of 1 to 2 mm and was gently pressed onto the mucosa to produce a cutting effect by using the Endocut mode with effect 2 (output 60 W). The proximal (oral) half of the mucosal incision was completed first, followed by the distal (anal) half. A retroflexed position was used to make the proximal incision, and a straight endoscope position was used for the distal incision.

3. Dissecting the submucosal layer beneath the lesion. Before incising all around the lesion, dissection of the submucosa was started from the area where the mucosal incision was made so as not to flatten the lifting area as time passed. The principle device used for submucosal dissection also was a flex knife, with the same length as for a mucosal incision when using the forced coagulation mode (output 40 W). Repeated injections of the submucosal injection solution were used to maintain the submucosal fluid cushion and to minimize the risk of perforation. The patient's position was regularly changed to facilitate visualization of the tissue plane, and dissection continued until the lesion was completely excised. Hemostatic forceps (SDB2422; Pentax) were used in the soft coagulation mode (output 50 W) to control any visible bleeding.

4. Treatment of artificial ulcer after ESD. After resection of the lesion, any visible vessels within the artificial ulcer were treated with hemostatic forceps in the soft coagulation mode (output 50 W), to prevent delayed bleeding. Finally, sucralfate (Ulcerlmin; Chugai) was sprayed onto the ulcer base, both to confirm hemostasis and to coat the surface of the ulcer.[10]

The lesion was successfully resected en bloc, without any complications (Fig. 4A to E). The total procedure time was approximately 270 minutes. On the following day, the patient was allowed clear fluids, followed by a light diet 48 hours after the procedure, and was eventually discharged a week after ESD. Repeat colonoscopy 2 months after ESD revealed only a small residual mucosal defect within the center of the ulcer scar, approximately 2 cm in diameter, without any stenosis of the colorectal lumen (Fig. 4F). The resected specimen measured 94 × 80 mm, with the tumor occupying an area of 91 × 72 mm (Fig. 5). Histologic assessment showed a tubulovillous adenoma with moderate to severe atypia and a well-differentiated adenocarcinoma, without any evidence of vessel infiltration or submucosal invasion. Both the lateral and vertical margins were free of tumor. At 1-year follow-up, there was no evidence of local recurrence or metastatic spread.

DISCUSSION

EMR, or an inject and cut technique, has become increasingly popular as a treatment option for node negative and flat- or depressed-type colorectal lesions.[11,12] This technique is simple and relatively straightforward for resecting small lesions; however, large, flat lesions, e.g., LSTs, require piecemeal resection, resulting in multiple fragments, which

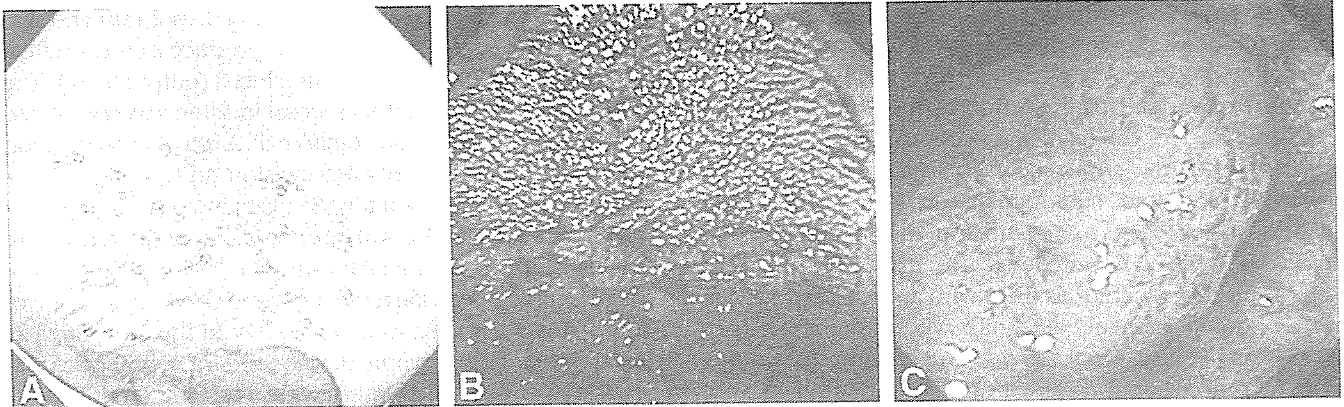

Figure 1. Colonoscopic views of the laterally spreading tumor. **A,** Original view: flat, carpet-like, white area at the rectosigmoid junction, with visible areas of nodularity and erythema. **B,** Chromoendoscopic view with indigo carmine dye, showing demarcation of the margin of the lesion. **C,** Magnifying view with crystal violet staining; the large nodule shows irregular pits indicative of malignancy.

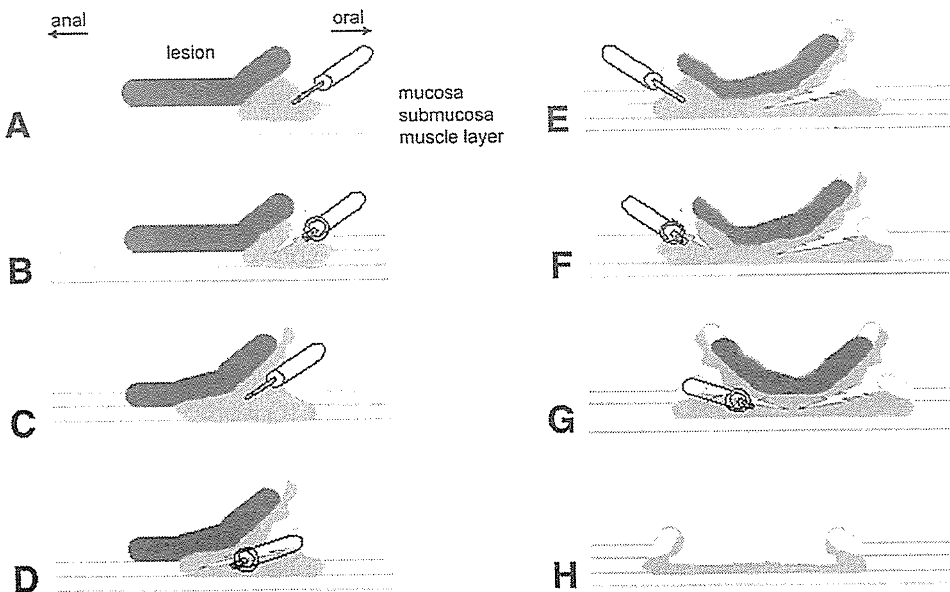

Figure 2. Schema of endoscopic submucosal dissection of our technique. **A,** Submucosal injection with a mixture of hyaluronic acid, glycerin, and sugar at the oral margin of the lesion, in the retroflexed position. **B,** Initial mucosal incision with a flex knife at the oral margin of the lesion. **C,** Additional submucosal injections to maintain the submucosal lift. **D,** Continuation of submucosal dissection with a flex knife from the oral margin to the center of the lesion. **E,** Submucosal injection at the anal margin with the endoscope in the straight position. **F,** Initial mucosal incision with a flex knife at the anal margin and extension of incision in a circumferential manner around the lesion. **G,** Continuation of submucosal dissection with a flex knife until detachment of the lesion; repetition of submucosal injections from the exposed submucosal layer to keep the lesion lifting from the muscle layer. **H,** Complete resection of the lesion in one piece.

makes accurate histologic evaluation difficult. To overcome this problem, large lesions are sometimes resected surgically, even when the lesion is limited to the mucosa, with the associated morbidity and mortality risks from surgery.

ESD has become a new modality of endoscopic therapy in the stomach[3-5] and has been developed from one of the EMR techniques, endoscopic resection after local injection of a solution of hypertonic saline solution and epinephrine.[13] The difference between EMR and ESD is that the latter technique involves dissection of the submucosal layer underneath the lesion by using electrosurgical knives. One of the advantages of ESD over EMR is that the shape and the size of the resected specimen can be controlled by the operator, and even a large lesion with a complicated shape can be resected as a single fragment. However, in contrast to the stomach, the lumen of the colon and the rectum is smaller and tortuous, or angulated. Furthermore, the

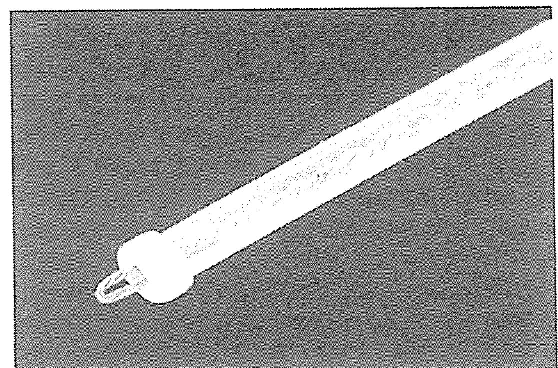

Figure 3. Flex knife. The knife consists of twisted looped wires. The tip of the outer sheath is rolled over to keep a constant cutting depth.

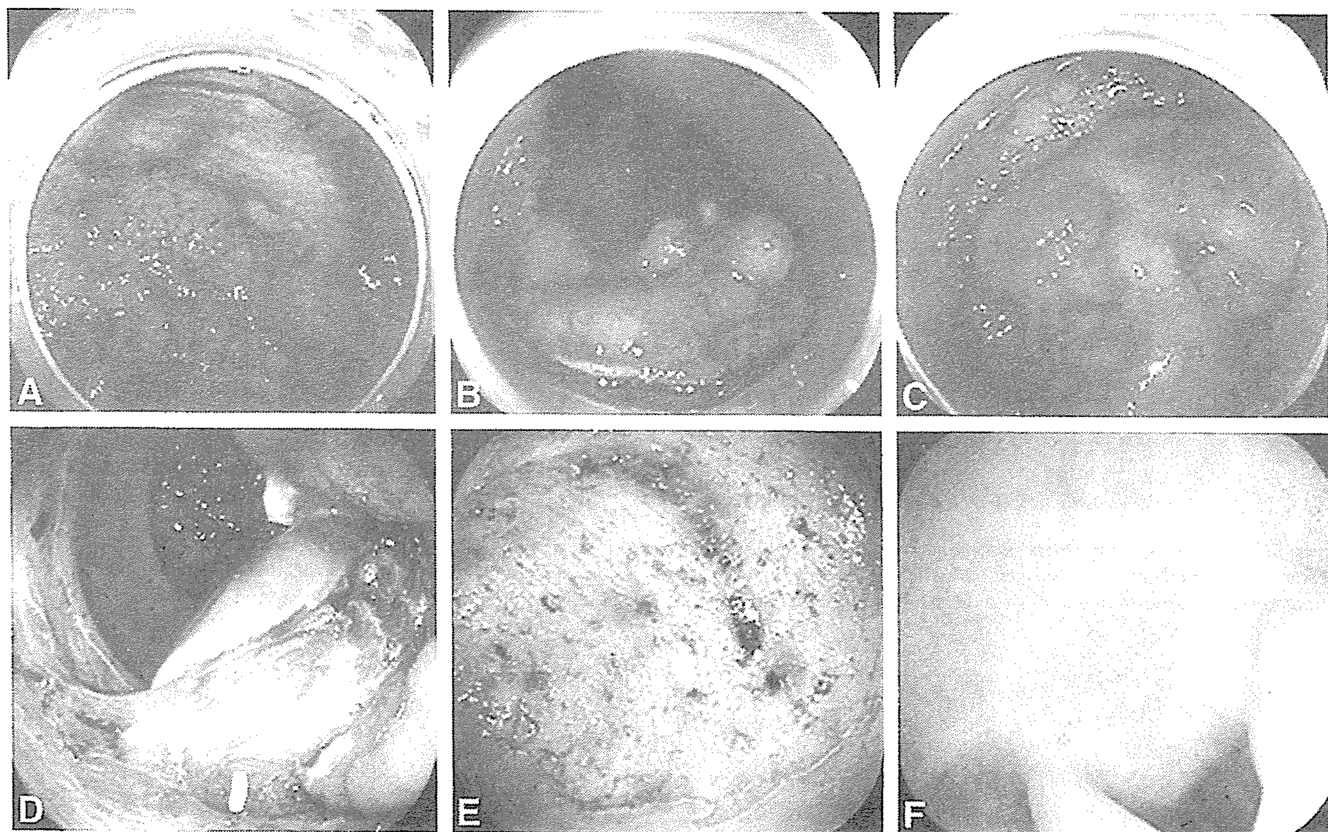

Figure 4. Endoscopic submucosal dissection of the laterally spreading tumor. **A,** Making a submucosal fluid cushion; a mixture of 1900 kDa hyaluronic acid and glycerin with fructose plus saline solution is injected into the submucosal layer to lift up the lesion from the muscle layer. **B,** Mucosal incision around the lesion; by using a flex knife, a mucosal incision is made from the oral side of the lesion. **C,** Submucosal dissection with circumferential mucosal incision; repetition of mucosal incision and submucosal dissection with a flex knife is continued until circumferential mucosal incision shrinks the lesion to the center. **D,** Submucosal connective tissue under the lesion; during submucosal dissection, changing the patient's position is very important to locate the connective tissue to cut. **E,** Artificial ulcer after removal; vessels on the ulcer base should be treated by hemostatic forceps to prevent delayed bleeding. **F,** Two months after removal; the ulcer is almost cured, with mild deformity.

thickness of the colorectal wall is only a few millimeters, and, within the colorectum, there are numerous bacteria. Because of the potential difficulties of performing ESD in the colorectum, we undertook a number of experiments to determine the safety and the feasibility of performing ESD in the colorectum. Firstly, we investigated which solutions were ideal for creating a submucosal fluid cushion that would be of sufficient thickness, as well as long lasting, so as to prevent colorectal perforation. The best solution was found to be a mixture of high-molecular weight hyaluronic acid with sugar and glycerin.[14,15] Secondly, in collaboration with Olympus, we developed a novel electrosurgical knife, the flex knife, which has the following characteristics: (1) a soft, thick, and looped distal tip to reduce the risk of perforation through the relatively thin colorectal wall; (2) an adjustable length of knife to control the depth of cutting; (3) a rolled tip on the outer sheath of the knife to maintain a constant cutting depth; and (4) a thin-caliber outer sheath to facilitate maneuverability of the knife.[16]

Lesions in the rectum and the distal sigmoid colon may be resected by using a variety of techniques, including laser therapy,[17] transanal surgery,[18] stereoptic microsurgery,[19] even use of the urologic resectoscope,[20] and now ESD. However, ESD has the advantage of both en bloc resection and that it can be performed with just topical anesthesia of the anal canal. Furthermore, the deepest part of the submucosal layer and the muscle layer remain intact, even after tumor resection, which may result in less deformity or stricturing of the colorectal lumen, as demonstrated in this case. Future studies should help to determine whether the morbidity and mortality risks from ESD are less than those associated with conventional abdominal surgery or minimally invasive techniques.

In summary, this case shows that ESD is a promising technique for the resection of large LSTs in the rectosigmoid junction. However, at present, colorectal ESD is technically difficult to perform, requires expertise, and further work and refinements in technique are necessary before ESD

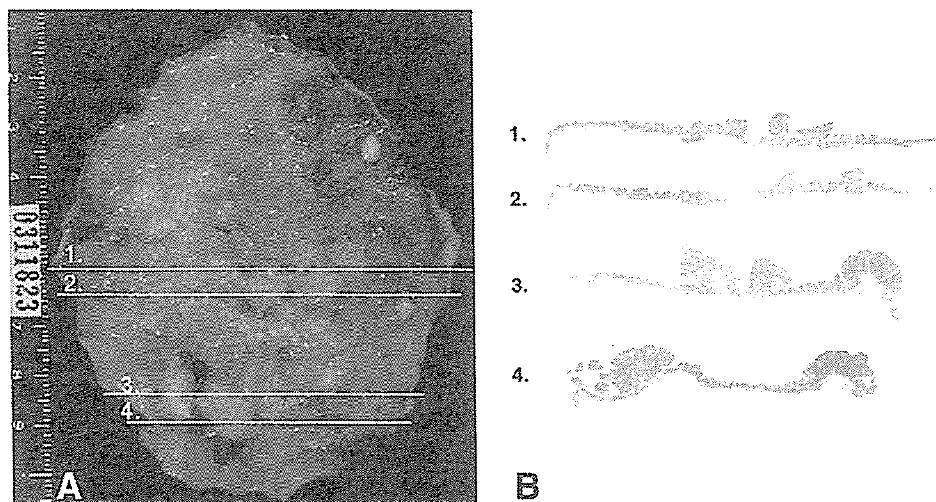

Figure 5. Resected specimen by endoscopic submucosal dissection. **A,** Macroscopic view of the lesion; the tumor is spread in the area of 91 × 72 mm, and some nodular changes are observed in the flat tumor. **B,** Loupe view of the lesion; the large nodules consist of well-differentiated adenocarcinoma, without vessel infiltration or submucosal invasion, and the flat area consists of tubulovillous adenoma, with moderate to severe atypia. (H&E, orig. mag. ×1).

may be accepted as a standard technique for the treatment of large intramucosal colorectal tumors.

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DISCLOSURE

The flex knife was produced in collaboration with Olympus Optical Co.

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Current affiliations: Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; Second Department of Internal Medicine, Wakayama Medical University, Wakayama, Japan.

Reprint requests: Mitsuhiro Fujishiro, MD, Department of Gastroenterology, Graduate school of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, Japan.

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ARTICLE

Mixed Gastric- and Intestinal-type Metaplasia Is Formed by Cells with Dual Intestinal and Gastric Differentiation

Toru Niwa, Yuzuru Ikehara, Hayao Nakanishi, Harunari Tanaka, Ken-ichi Inada, Tetsuya Tsukamoto, Masao Ichinose, and Masae Tatematsu

Division of Oncological Pathology, Aichi Cancer Center Research Institute, Nagoya, Japan (TN,YI,HN,HT,K-iI,TT,MT) and Second Department of Internal Medicine, Wakayama Medical College, Wakayama City, Japan (TN,MI)

SUMMARY We have proposed to divide intestinal metaplasia (IM) into two categories, i.e., a mixed gastric and intestinal (GI) type, and a solely intestinal (I) type, based on the residual gastric phenotype cells. The GI-mixed-type IM can be identified by the presence of both cells with either gastric or intestinal phenotypes in a single gland. This study is conducted to elucidate whether cells in the GI-mixed-type IM glands can simultaneously present both gastric and intestinal phenotypes. MUC5AC, MUC2, CD10 and villin expressions were investigated in 20 samples from five gastric cancer cases, directly using either AlexaFluor 488- or 568-labeled specific monoclonal antibodies and observed by fluorescent microscopy and confocal laser-scanning microscopy. GI-mixed IM glands comprise a population expressing MUC5AC and MUC2, MUC5AC and villin, and MUC5AC and CD10. MUC2 and villin expressions were reciprocally increased with decreasing MUC5AC expression, while CD10 expression was limited to cells with only a residual MUC5AC expression or no expression. These results suggest that a heterogeneous cell population with both gastric and intestinal phenotypes would develop into a single intestinal phenotype, as reflected in the progression of intestinal metaplasia from GI-mixed-type- to I-type IM-type glands.
(J Histochem Cytochem 53:75–85, 2005)

KEY WORDS
intestinal metaplasia
MAC5AC
MUC2
villin
CD10
human stomach

INTESTINAL METAPLASIA (IM) is histologically defined by the presence of intestinal-type cells such as goblet, Paneth, and absorptive cells and is often encountered in chronic and/or atrophic gastritis. IM has long been widely believed to be a premalignant condition associated with a differentiated adenocarcinoma genesis (Morson 1955; Stemmermann and Hayashi 1968; Sugimura et al. 1982; Filipe et al. 1985; Correa 1992; You et al. 1993). As the various histological features of IM glands are well known, efforts have been made to distinguish IM glands morphologically and/or enzyme histochemically to elucidate which typical premalignant aspects might be associated with differentiated adenocarcinomas (Kawachi et al. 1974; Teglbjaerg and Nielsen 1978; Jass and Filipe 1979; Matsukura et al. 1980; Segura and Montero 1983; Filipe et al. 1988; Correa 1992; Jass and Walsh 2001; Silberg et al. 2002). For example, IM has been recognized as either a complete or an incomplete type IM, or as a small- (Type I and Type II) or large- (Type III) intestinal-type IM (Kawachi et al. 1974; Matsukura et al. 1980; Filipe et al. 1985, 1988; Matsukuma et al. 1990). Though these classifications have been generally accepted, they have overemphasized the characteristics common to cells in the small intestine, while neglecting to take into account the preserved gastric phenotype. In contrast, we have proposed a new classification of IM based on the presence or absence of gastric-type cells in IM glands, which we have subdivided into two major types, i.e., a mixed gastric and intestinal type (GI-mixed-type) and a solely intestinal type (I-type) (Inada et al. 1997, 2001).

According to this classification, I-type IM glands are solely comprised of intestinal phenotypic cells, whereas GI-mixed-type IM glands also contain gastric phenotypic cells. Interestingly, although the number of

Correspondence to: Yuzuru Ikehara MD, PhD, Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan. E-mail: yikehara@aichi-cc.jp
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gastric cells varies as much as those in a gastric-predominant or an intestinal-predominant type, GI-mixed-type IM glands appear to gradually become I-type IM glands (Inada et al. 1997; Tatematsu et al. 2003). The exact mechanisms by which these two kinds of phenotype cells or phenotype shifts come to be produced have yet to be determined, but at least two explanations are possible. The first is that aberrantly expressed Cdx1 and/or Cdx2 transcriptional factors, mammalian homologs of the caudal-related homeobox genes, may play an important role in such processes (Silberg et al. 1997; Mizoshita et al. 2001; Tsukamoto et al. 2003; Yuasa 2003). The other would be that some genetic alterations such as methylation occur in stem cells leading them to supply such various cell types, especially as seen in GI-mixed-type IM in our classification, resulting in the phenotype change caused by their accumulation (Kang et al. 2001,2003a,b; Kim et al. 2004; Lee et al. 2004).

Immunohistochemical techniques are widely used to identify intestinal and gastric cell differentiation for the classification of gastric cancers and IM (Inada et al. 1997; Reis et al. 1999; Inada et al. 2001; Jass and Walsh 2001; Mizoshita et al. 2001; Silva et al. 2002; Kawachi et al. 2003; Tatematsu et al. 2003). To date, a preferred method to evaluate IM and gastric cancers utilizes the anti-mucin core proteins 5AC (MUC5AC) and 6 (MUC6) together with anti-CD10, anti-villin, and anti-MUC2 antibodies. Mucin core proteins comprise an expanded gene family consisting of at least 19 members (Tanaka et al. 1991; Gum et al. 2002; Chen et al. 2003; Ringel and Lohr 2003), of which *MUC5AC*, *MUC6* and *MUC2* genes are homologous to each other and are localized at chromosome 11p15.5 within a 400-kbp gene span (Pigny et al. 1996; Winterford et al. 1999). Their expressions might be differentially regulated by the restricted MUC5AC presence on surface epithelial cells (Reis et al. 1997), MUC6 on cells in the glandular compartment of pyloric mucosa (Ho et al. 1995; Reis et al. 2000), and MUC2 in the goblet cells of small and large intestines, and on IM (Jass 2000). A secreting endopeptidase, CD10 (Landry et al. 1994; Sezaki et al. 2003), and one of the actin-binding cytoskeletal proteins, villin (Landry et al. 1994; MacLennan et al. 1999; Pinto et al. 1999), are also observed in intestinal cells, whose expressions indicate absorptive-cell differentiation in IM (Landry et al. 1994). Expressions of these molecules are widely used to evaluate gastric cancers whether the differentiation direction is toward gastric or intestinal phenotype.

Using an adaptation of this approach to investigate IM glands, it has been demonstrated that small populations of MUC2-positive cells containing either MUC5AC or MUC6 are present in IM glands (Ho et al. 1995; Inada et al. 1997,2001; Reis et al. 1999,2000; Tatematsu et al. 2003). However, it remains unclear whether these glands, probable GI-mixed IM glands, are composed of dual phenotype cells with intestinal and gastric differentiation. In the present study we evaluated the co-expression of MUC5AC and MUC2, MUC5AC and villin, and MUC5AC and CD10 in GI-mixed-type IM glands using multiple immunofluorescent staining techniques at the single-cell level. The combined use of these markers succeeded in providing evidence of cells with both gastric and intestinal phenotypes in IM.

Materials and Methods

Tissue Samples

Twenty normal stomach tissue samples were obtained from five patients with gastric cancer who underwent gastrectomy at Aichi Cancer Center Hospital. They were cut from normal areas more than 10 cm away from the cancer, immediately frozen in Tissue-Tek OCT (Optimal Cutting Temperature) Compound (Sakura Finetechnical Co. Ltd.; Tokyo, Japan) with liquid nitrogen and then stored at −80C until use. Four-μm-thick frozen sections prepared with a cryostat were fixed in cold methanol and dried at room temperature for use in immunohistochemical analysis.

Antibodies

Table 1 shows the characteristics of mouse monoclonal antibodies (MAbs) used in this study. To specifically detect the immune reactions with two respective mouse MAbs, we employed Zenon Mouse IgG-labeling kits to directly label the MAbs with either AlexaFluor 488 or AlexaFluor 568 (Molecular Probes; Eugene, OR). Fluor-labeled MAbs were prepared immediately prior to use, according to the suppliers' protocols. The optimal concentrations of primary MAbs were determined empirically, and the final concentrations were 1:100 of anti-MUC5AC, 1:100 of anti-CD10, and 1:5000 of anti-villin MAbs. In all cases, isotype-matched monoclonal antibodies were used as a negative control.

Immunofluorescent Staining

Four μm-thick frozen sections were fixed in cold methanol for 10 min, then air-dried at room temperature for 30 min and rehydrated in PBS for 15 min at room temperature. To reduce nonspecific bindings, the sections were incubated with a blocking reagent (PBS containing 0.2% Triton X-100, 0.2% BSA, and 5% heat-inactivated normal goat serum) for 30 min at room temperature and then reacted with a mixture of two primary antibodies labeled with either AlexaFluor 488 or 568 for 2 hr at room temperature. After washing twice with PBS containing 0.2% Triton X-100 for 15 min,

Table 1 Antibodies used in this study

Antigen	Clone	Reactivity	Dilutions
MUC5AC	CLH2	Gastric foveolar cells	1:100
MUC2-NCL	Ccp58	Goblet cells	1:100
Villin	BDID2C3	Brush border	1:5000
CD10	56C6	Brush border	1:100

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the sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes) to stain the nucleus for 1 min at a dilution of 1:20000. After washing with PBS, the sections were mounted in Mowiol 4-88 Reagent (CALBIOCHEM; San Diego, CA). To remove O-linked glycosylation on MUC2 for Ccp58 anti-MUC2 antibody to bind the epitope, we performed alkali-catalyzed β-elimination on the frozen section, the same as previously reported (Hong and Kim 2000).

Immunofluorescence and Confocal Laser-scanning Fluorescence Microscopy

Multicolor-stained tissues with AlexaFluor 488, AlexaFluor 568, and DAPI were observed using an Olympus BH5 fluorescence microscope (Olympus; Tokyo, Japan) equipped with a xenon arc lamp and an appropriate filter set. Confocal laser scanning was performed with the Radiance 2100 K-3 system (BioRad; Clinisciences S.A., Montrouge, France) that employs optical fibers both in the illumination source and the detection aperture. This system was equipped with a 50-mW Crypton-Argon laser and filters allowing excitation with both a 488-nm and a 560-nm laser line. Two channels were available for simultaneous data acquisition: Channel 1 (displayed as green) could use a 510–550-nm bandpass filter and either a 515-nm or a 530-nm longpass filter, while Channel 2 (displayed as red) could include either a 550-nm or 590-nm longpass filter.

Image Analysis

All images were recorded by a digital video camera and converted to TIFF files. Merged images were made using Adobe Photoshop software (Adobe Systems; San Jose, CA).

Results

Since all of the monoclonal antibodies used in this study were IgG1, we employed a Zenon antibody-labeling kit to detect two antigens simultaneously. This technique makes it possible to individually detect several co-expressed antigens when using the same isotype antibodies. We conjugated anti-MUC2 mAb, anti-villin mAb and anti-CD10 MAb with AlexaFluor 488, while anti-MUC5AC was conjugated with AlexaFluor 568 (anti-CD10 MAb was also conjugated with AlexaFluor 568 in some cases). Their specific bindings (Table 1) were not modified by the Zenon-labeling procedure.

Co-expression of MUC2 and MUC5AC on GI-mixed-type IM

As shown in Figure 1, intestinal and gastric differentiation makers were detected in IM glands. In the first series of experiments using such specimens, we examined the expression of MUC2 as a marker for intestinal goblet cells and that of MUC5AC as a marker for gastric-surface columnar cells. Surface columnar epithelial cells exhibited MUC5AC without MUC2 expression in normal gastric mucosa, while goblet cells of the intestinal or I-type IM exhibited MUC2 without MUC5AC, as demonstrated in previous studies (Figures 2A and 2C) (Inada et al. 1997,2001; Tatematsu et al. 2003). GI-mixed-type IM glands, which were identified by the presence of both MUC5AC and MUC2 in a single gland (Figure 2B), demonstrated both the antigens in glands with a differential positive cell ratio. MUC2-positive cells showed a goblet cell-like feature with MUC5AC expression that included the MUC2 epitope in their cytoplasm of determined GI-mixed-type IM glands (Figure 2B). This result indicates that GI-mixed-type IM glands include cells sharing both gastric and intestinal phenotypes. Interestingly, the co-expressed MUC2 and MUC5AC epitope was not completely colocalized in their cytoplasm. To examine the differential cellular localization between MUC2 and MUC5AC in GI-mixed-type IM cells in more detail, we used a confocal laser-scanning microscope. MUC2 was clearly observed at the center of the cytoplasm, which was surrounded by the MUC5AC-expressing area (Figures 3A–3F), suggesting that MUC2 and MUC5AC are sorted in a differential manner.

Furthermore, MUC2 antigen appeared in the supra-nuclear region of goblet cells in I-type IM glands (Figure 2C), whereas its distribution appeared more diffusely in the mucous vesicle of GI-mixed type IM cells (Figure 2B). The alteration in MUC2 distribution seems to arise from the difference between I-type IM and GI-mixed-type IM cells. To elucidate the possibility that higher glycosylation reduced antibody binding in goblet cells of I-type IM the same as in the colonic mucosa previously described (Hong and Kim 2000), alkali-catalyzed β-elimination was performed to remove glycosylation. Following the procedure, increased anti-MUC2 MAb reactivity was observed, which was MUC2 staining similar to that in GI-mixed-type IM glands (Figures 3G and 3H). These results suggest that MUC2 in I-type IM cells is more abundantly glycosylated than in GI-mixed-type IM. The cellular distribution of MUC5AC showed no apparent changes between GI-mixed-type IM cells and normal gastric surface columnar cells.

Co-expression of MUC5AC and Villin/CD10 in GI-mixed-type IM

In a second set of experiments, we examined MUC5AC as a marker for gastric surface columnar cells and either villin or CD10 as the other markers for intestinal cells to verify whether the GI-mixed-type IM glands exhibit both intestinal and gastric phenotypes. Both villin and CD10 have been widely used as specific markers to identify intestinal absorptive cells and are exclusively expressed at the brush borders of cells solely in I-type IM glands but not in gastric glands (Figures 4 and 5). GI-mixed-type IM glands were identified by the presence of MUC5AC and villin in individual glands (Figure 4). Villin was detected on the

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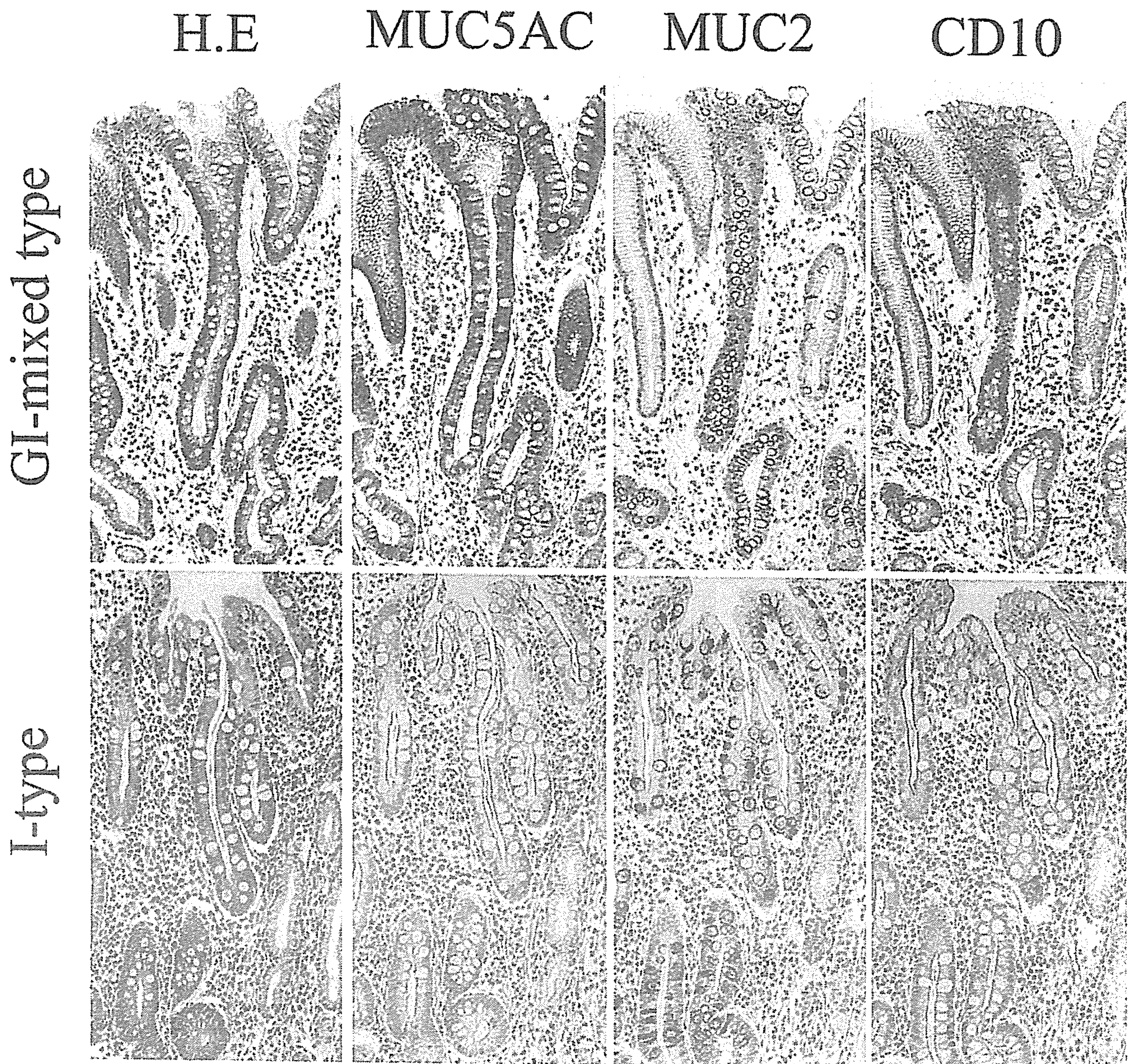

Figure 1 Immunnohistochemical analysis for IM glands with sequential sections. IM glands were detected by the expression of either CD10 or MUC2. Solely I-type IM glands express the intestinal markers without MUC5AC expression (lower panel, ×40), while GI-mixed-type IM glands express the intestinal markers with MUC5AC (upper panel, ×40).

apical end of MUC5AC-positive cells in GI-mixed-type IM glands, whose expressed cell numbers increased with diminishing MUC5AC expression in the glands. It is noteworthy that CD10 expression was not always detected with villin at the brush border in GI-mixed IM glands (Figure 6) but was also occasionally localized in the cytoplasm (Figure 5). Its inclusion body-like appearance was the same as that reported in familial microvillus-inclusion body disease (Groisman et al. 2002). This inclusion body-like CD10 staining pattern is more apparent in GI-mixed-type IM glands with more abundant MUC5AC-expressing cells, but it disappears with declining MUC5AC expression.

Discussion

To date, several immunohistochemical studies have demonstrated an intestinal or a gastric phenotype by the detection of specific molecular expressions in IM glands

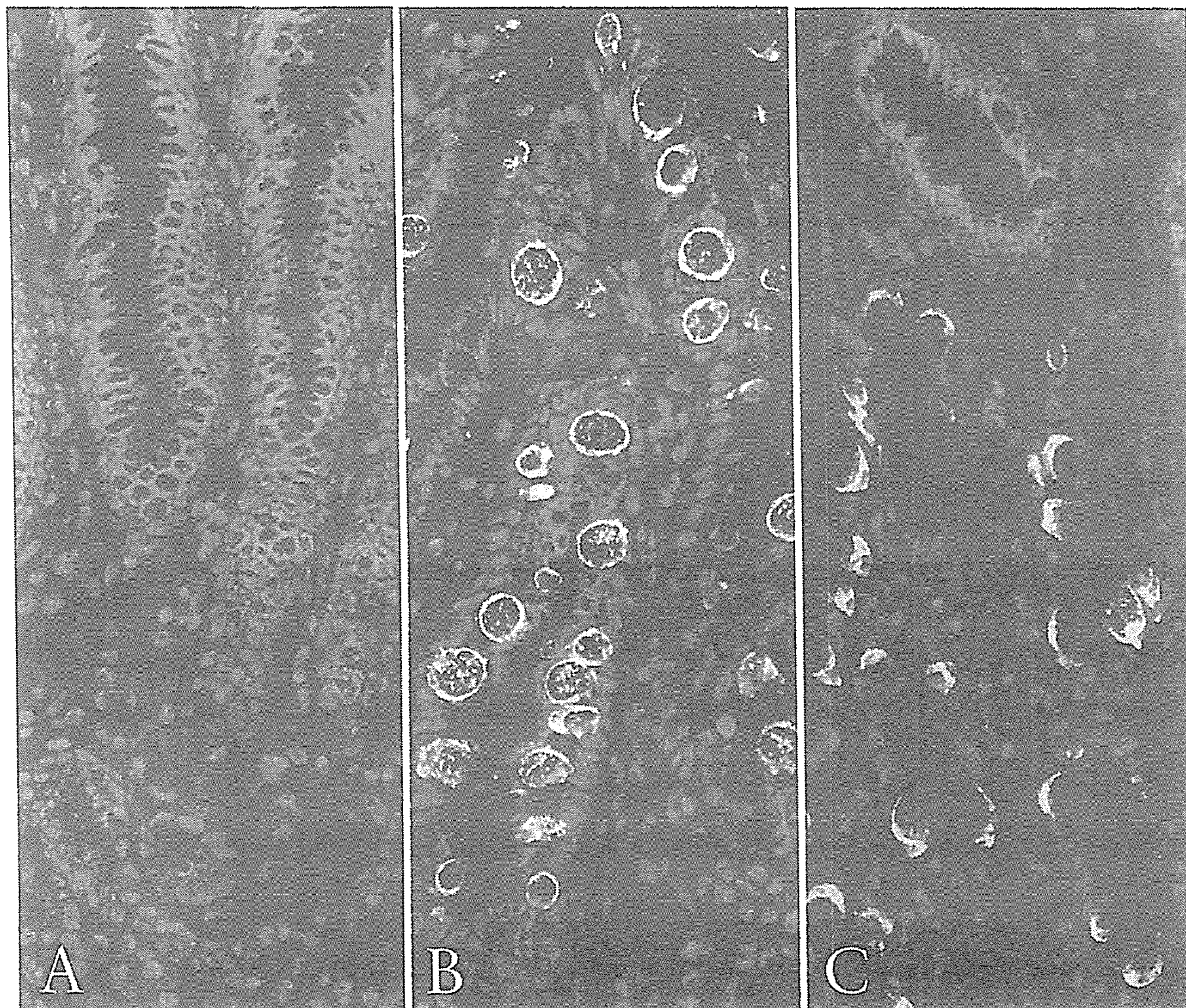

Figure 2 Localization of MUC5AC and MUC2 mucin core protein in normal gastric glands and IM glands. Frozen sections were stained with two mouse MAbs against MUC5AC directly labeled with AlexaFluor 568 and MUC2 with AlexaFluor 488 as described in Materials and Methods. Nuclei were counter-stained with DAPI (blue) and observed by fluorescence microscopy. MUC5AC (red) and MUC2 (green) mucin core protein expressions were observed in the cytoplasm of foveolar epithelial cells in normal gastric mucosa (**A**) and in the Golgi apparatus of goblet cells in solely I-type IM glands (**C**), respectively. Colocalization of MUC5AC and MUC2 on the same cells was demonstrated by merged images (yellow) in GI-mixed-type IM glands (**B**, ×60).

using serial sections (Ho et al. 1995; Inada et al. 1997, 2001; Reis et al. 1999; Jass 2000; Shaoul et al. 2000; Silva et al. 2002; Tatematsu et al. 2003). These studies have attempted to demonstrate that some cells are either intestinal or gastric phenotypic markers, suggesting limited possibilities for the same cells to have both gastric- and intestinal-marker antigens (Reis et al. 1999, 2000; Lopez-Ferrer et al. 2000,2001; Shaoul et al. 2000). In the present study, we were able to overcome such limitations by employing Zenon antibody-labeling technology, thus successfully demonstrating the coexpression of both gastric and intestinal molecular markers in the same cells in GI-mixed-type IM glands, in which MUC5AC clearly existed with MUC2, or vil-

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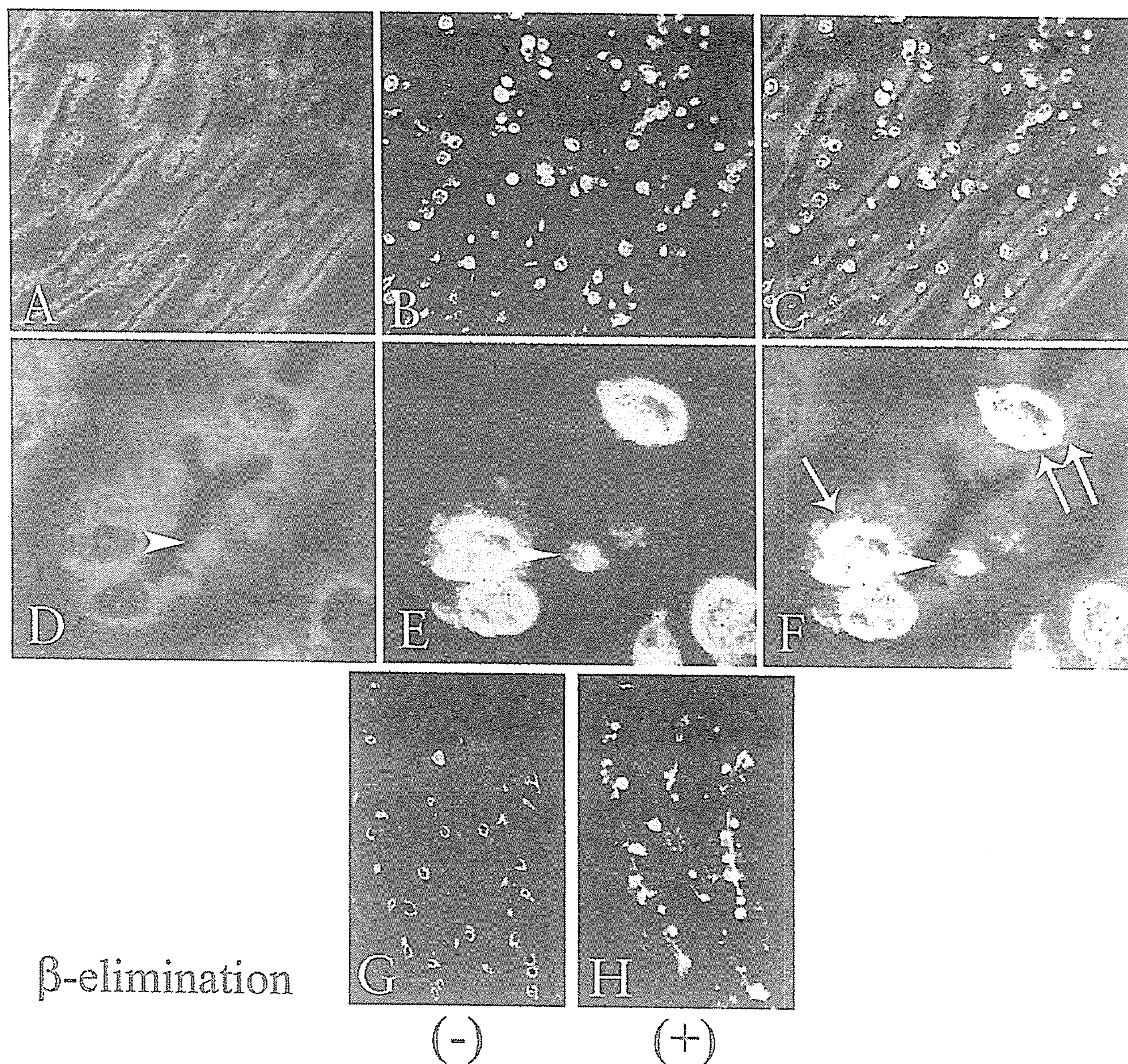

Figure 3 Colocalization of MUC5AC and MUC2 mucin core proteins in GI-mixed-type IM glands. Frozen sections were stained by the double-immunofluorescence method using two mouse MAbs against MUC5AC and MUC2 directly labeled with AlexaFluor 568 or 488. MUC5AC (red) and MUC2 (green) expressions in GI-mixed-type IM glands were observed by confocal laser scanning microscopy (**A–C**: low-power views; **D–F**: high-power views). (**A,D**) MUC5AC was observed in the cytoplasm of almost all columnar epithelial and goblet cells, except for the center of a secretory vesicle of goblet cells. (**B,E**) MUC2 expressions were observed in the Golgi area in columnar cells (arrowhead) and the secretory vesicles in goblet cells. (**C,F**) Colocalization of MUC5AC and MUC2 was seen on the periphery of secretory vesicles in the composite images (yellow, arrow). (**G,H**) β-elimination increased MUC2 antibody (Ccp58) reactivity in mucous vesicle of solely I-type IM glands. (**A–C,G,H** ×20), (**D–F**, ×60).

lin and/or CD10. These findings suggest that mixed gastric and intestinal type metaplasia is formed by cells with dual differentiation and are consistent with the previously demonstrated evidence that some metaplastic cells have both intestinal and gastric differentiation-specific structures (Goldman and Ming 1968).

The cells in GI-mixed-type IM glands exhibited MUC2 with MUC5AC in their cytoplasm (Figures 2 and 3), while the cells in I-type IM glands exhibited MUC2 in the peri-nuclear Golgi apparatus area. The subcellular localization of MUC2 appeared to shift from secretion vesicles to the Golgi apparatus area with a

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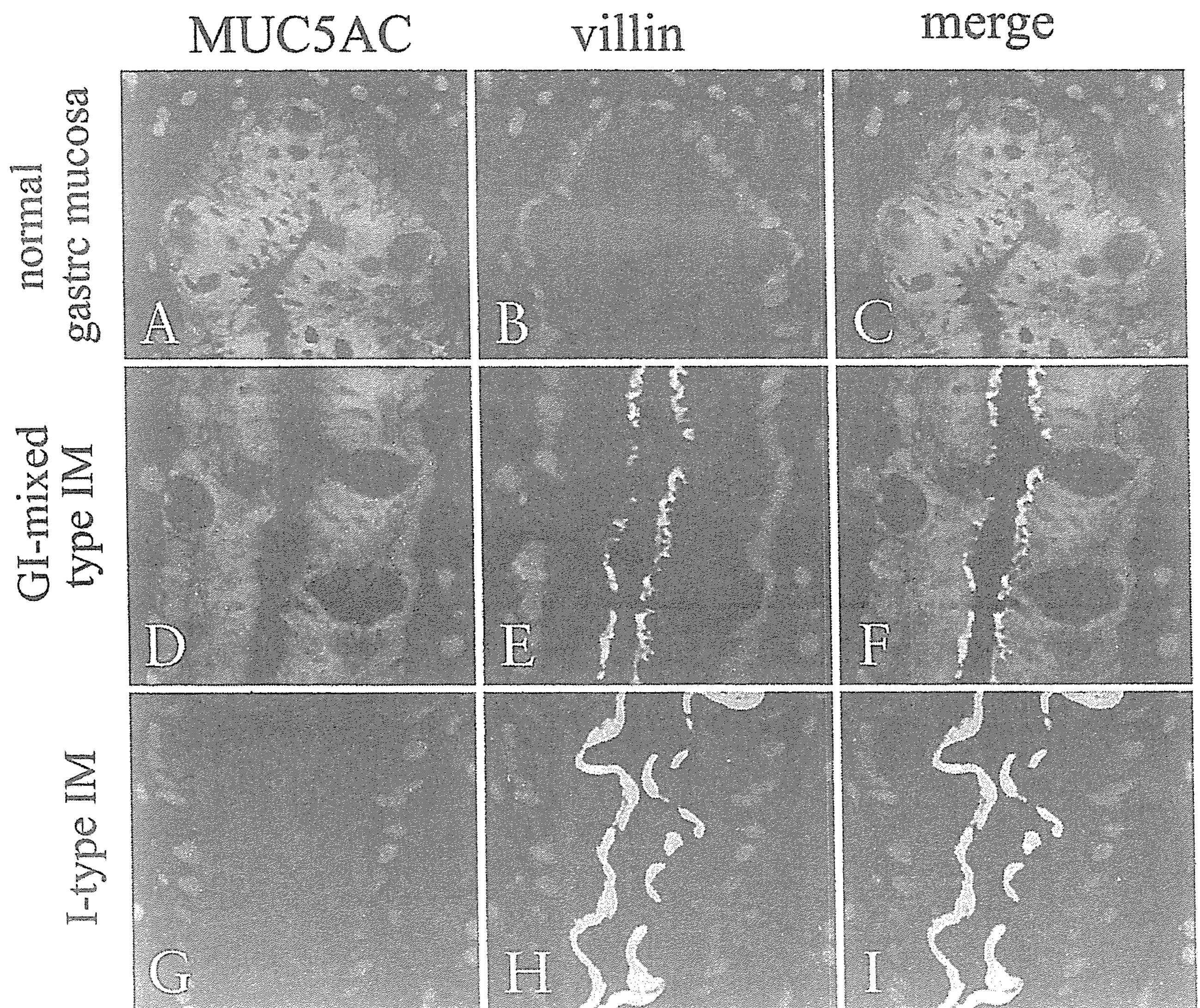

Figure 4 Expression of MUC5AC and villin in normal gastric glands and GI-mixed-type IM glands. Frozen sections were stained by the double-immunofluorescence method using two mouse MAbs against villin and MUC5AC directly labeled with AlexaFluor 488 or 568. Nuclei were counter-stained by DAPI (blue) and observed by fluorescence microscopy. (A–C) Only MUC5AC (red) without villin (green) was seen in gastric columnar epithelial cells. (G–I) Such cells in I-type IM glands exhibited only villin without MUC5AC. (D–F) MUC5AC was observed in the cytoplasm and villin of the micro-villi of the columnar cells in GI-mixed-type IM glands. Composite images demonstrate the absence of any yellow signal in the cells, suggesting their different subcellular localization (F). (A–I, ×60).

histological alteration from GI-mixed-type IM to I-type IM. A glycosylation change might be one of the probable explanations, since the MUC 2 antibody used in this study could detect only underglycosylated MUC2 core protein for the epitope as discussed in previous studies (Hong and Kim 2000; Shaoul et al. 2000). In the present study we observed the enhanced anti-MUC2 antibody staining in I-type IM glands by alkali-catalyzed β-elimination, which was similar to the MUC2 staining on GI-mixed-type IM glands. This result indicates that O-linked glycosylation limited the detection of MUC2 expression in goblet cells in I-type IM glands, suggesting that the difference between the higher glycosylated MUC2 in I-type IM glands and the lower glycosylated MUC2 in the goblet cells in the GI-mixed-type IM glands would be the other maturation indicator for goblet cells in IM glands.

The columnar epithelial cells, similar to the intestinal absorptive cells, were seen in GI-mixed-type IM glands that exhibited MUC5AC and either villin or CD10. Compared with villin-positive cells, CD10 was preferentially found in the faint MUC5AC-preserved columnar cells, suggesting that CD10 exists in cells of the more intestinalized GI-mixed-type IM glands (Fig-