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Questionnaire-Based Survey Conducted in 2011 concerning Endoscopic Management of Barrett's Esophagus in East Asian Countries

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Key Words
Barrett's esophagus · Diagnosis · Endoscopy

Abstract
Background/Aims: Endoscopic definitions and management of Barrett's esophagus vary widely among countries. To examine the current situation regarding diagnosis, epidemiology, management and treatment of Barrett's esophagus in East Asian countries using a questionnaire-based survey. ***Methods:*** Representative members of the Committee of the International Gastrointestinal Consensus Symposium developed and sent a questionnaire to major institutions in China, South Korea, Japan, Thailand, Indonesia, and the Philippines. ***Results:*** A total of 56 institutions in the 6 countries participated in the survey. We found that the presence of specialized columnar metaplasia is considered to be important for diagnosing Barrett's esophagus in East Asian countries except for Japan. C&M criteria have not been well accepted in East Asia. The palisade vessels are mainly used as a landmark for the esophagogastric junction in Japan. The prevalence of long segment Barrett's esophagus is extremely low in East Asia, while the prevalence of short segment Barrett's esophagus is very high only in Japan, likely due to

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different diagnostic criteria. ***Conclusion:*** Among East Asian countries, we found both similarities and differences regarding diagnosis and management of Barrett's esophagus. The findings in the present survey are helpful to understand the current situation of Barrett's esophagus in East Asian countries.

Introduction

Barrett's esophagus (BE) is thought to develop as a complication of chronic gastroesophageal reflux disease (GERD) and a major predisposing factor of esophageal adenocarcinoma. An increase in patients with GERD has been noted in recent years in Asia [1, 2], resulting in concern that the incidence of BE and esophageal adenocarcinomas arising in BE could also increase in Asian countries. To date, the reported prevalence of BE is lower in Asia in contrast to other parts of the world and most of those cases are short segment BE (SSBE) [3–5], as compared to long segment BE (LSBE), which is more commonly seen in Western countries. However, there is increasing evidence that the prevalence of BE and Barrett's adenocarcinoma is gradually rising in some parts of Asia [6]. Therefore, management of BE is a key issue in East Asian countries, though the current situation regarding the endoscopic management in each country is largely unknown. Moreover, definitions, concepts and opinions regarding BE vary widely among gastroenterologists and endoscopists in different countries [7].

While BE is an increasingly significant health problem worldwide, there remains a great deal of controversy, because of the absence of a universally and internationally accepted definition and grading system [8]. Furthermore, endoscopic landmarks for the esophagogastric junction (EGJ) have not been standardized, thus there is significant interobserver variability when determining the length of BE [9, 10], resulting in a lack of credibility regarding the reported prevalence rates.

There is increasing evidence showing the effectiveness of image-enhanced endoscopy (IEE), including narrow band imaging (NBI) with magnification endoscopy, autofluorescence imaging (AFI), and chromoendoscopy [11, 12], for the diagnosis of BE and Barrett's adenocarcinoma. As compared with conventional white light endoscopy with a blind four-quadrant biopsies as recommended by the American College of Gastroenterology (ACG) [13], the use of IEE may improve detection of subtle mucosal irregularities and facilitate targeted biopsies [14]. However, whether these modalities are readily available in all parts of East Asia is unknown.

The rising incidence of Barrett's adenocarcinoma has focused attention on preventing cancer by removing dysplasia and allowing normal squamous esophageal mucosa to regenerate. As a result, endoscopic esophageal mucosal ablative techniques, such as radiofrequency ablation (RFA), photodynamic therapy (PDT), and cryotherapy, have been utilized for advanced Barrett's lesions especially in Western countries [15–17]. Additionally, endoscopic mucosal resection techniques for dysplastic lesion in BE, such as endoscopic submucosal dissection (ESD) and endoscopic mucosal resection (EMR), have been employed to achieve potentially curative removal of Barrett's mucosa, which also allows for histological examinations of resected specimens and reduces morbidity associated with surgical esophagectomy [18, 19]. However, there is scant information regarding which techniques endoscopists most often choose for treatment of high grade dysplasia and mucosal cancer in patients with BE in various countries.

The aim of this study was to examine the current situation regarding diagnosis, epidemiology, management, and treatment of BE in East Asian countries by means of a questionnaire-based survey. In addition, differences and problems regarding management of such cases that exist among the queried countries were also analyzed.

Subjects and Methods

Subjects

Gastroenterologists and endoscopists at major institutions in China, South Korea, Japan, Thailand, Indonesia, and the Philippines participated in this survey. Only one gastroenterologist in each institution was expected to answer a questionnaire regarding endoscopic management of BE as the representative opinion of each institution.

Methods

This is the first questionnaire-based survey concerning endoscopic management of BE and Barrett's adenocarcinoma conducted by the International Gastrointestinal Consensus Symposium (IGICS), which is the international section of the Japanese Gastroenterological Association. Representative members from the IGICS committee provided a questionnaire to major institutions in each country, starting at the beginning of July 2011. Responses were collected until the end of December 2011. Each contained 33 questions focused on the following items: (1) diagnosis of BE; (2) epidemiology of BE and Barrett's adenocarcinoma; (3) management of BE; (4) advanced endoscopic imaging for diagnosis of BE, and (5) treatment of dysplastic lesions of BE. The contents of the questionnaire are described in the Appendix.

Table 1. Participating institutions

Country	Number	Cases of EGD in most recent year		
		<1,000	1,000–5,000	>5,000
China	6	0	2	4
Korea	10	0	0	10
Japan	15	0	7	8
Thailand	7	1	5	1
Indonesia	6	5	1	0
Philippines	12	4	8	0
Total	56	10	23	23

Table 2. Criteria used for diagnosis of Barrett's esophagus

Country	Criteria for diagnosis of Barrett's esophagus			
	SCE	squamous islands	esophageal glands proper	double layer of MM
China	100	83.3	83.3	16.7
Korea	100	60.0	10.0	10.0
Japan	40.0	93.3	33.3	33.3
Thailand	100	85.7	42.9	14.3
Indonesia	50.0	100	50.0	0
Philippines	91.7	91.7	41.7	8.3

We asked about the usage of criteria for diagnosis of Barrett's esophagus, with the following presented as options: specialized columnar epithelium (SCE), squamous islands confirmed by endoscopy, esophageal glands proper, and double layer of muscularis mucosae (MM) shown by histology. Values shown indicate the percentage of institutions using the indicated criteria in each country.

Table 3. Landmarks using to identify the EGJ

Country	EGJ landmark		
	gastric folds	palisade vessels	both
China	0	16.7	83.3
Korea	10.0	10.0	80.0
Japan	13.3	60.0	26.7
Thailand	50.0	0	50.0
Indonesia	16.7	0	83.3
Philippines	10.0	10.0	80.0

We asked about endoscopic landmarks used to identify the esophagogastric junction (EGJ) in each country, with the following presented as options: upper end of gastric folds, lower end of esophageal palisade vessels, and both. Values shown indicate the percentage of institutions using the indicated landmarks.

Results

Participating Institutions

In total, 56 institutions in 6 countries participated in this survey. The numbers of participating institutions in each country and cases of esophago-gastric-duodenoscopy (EGD) encountered in the most recent year are shown in table 1. More than 3,000 EGD examinations were performed in the most recent year at over the half of the surveyed institutions.

Diagnosis of BE

The definition of BE differs throughout the world [7, 20]. To elucidate current opinions regarding its definition in East Asia, we enquired about the use of criteria for diagnosis of BE, with the following 4 presented as options; specialized columnar epithelium (SCE), squamous islands confirmed by endoscopy, esophageal glands proper, and double layer of muscularis mucosae (MM) shown by histology findings. In terms of SCE, the percentage of institutions using the presence of SCE as a criterion was below 50% only in Japan (table 2), while the majority of endoscopists in the other countries accepted that for diagnosis of BE. Notably, the presence of squamous islands in columnar epithelium confirmed by endoscopy was accepted for diagnosis of BE in all of the surveyed countries. On the other hand, evidence of esophageal glands proper confirmed by histology was thought to be not suitable for diagnosis of BE in all except China. Likewise, a double layer of MM was thought to be unfitted for diagnosis of BE in all of the surveyed countries.

The next important issue is endoscopic classification of BE. The Prague C&M criteria were proposed in 2004 as a universal standard for endoscopic diagnosis of BE [21]. However, there is no information regarding whether those are widely used for endoscopic diagnosis of BE in East Asia. Thus, we asked about the use of the C&M criteria in each country. As shown in figure 1, C&M criteria are not used as the primary standard for endoscopic diagnosis of BE in any of the countries. Moreover, 10–20% or more of endoscopists in the participating institutions did not even know those criteria. In the C&M criteria, the proximal end of the gastric folds is considered to be the primary landmark for the EGJ, while another available landmark is the esophageal palisade vessels [22]. Thus, we also asked about the endoscopic landmark used to identify the EGJ in each country. Both the gastric folds and palisade vessels were used for identification of the EGJ in most of the surveyed countries (table 3). Interestingly, the palisade vessels are used as the main landmark only in Japan.

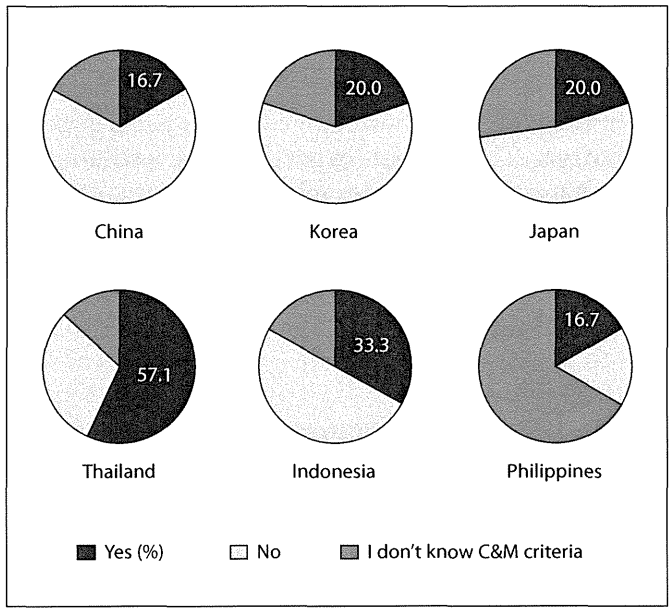

Fig. 1. Use of C&M criteria for endoscopic diagnosis of Barrett's esophagus. We asked about use of the C&M criteria in each country and the answers for each country are presented as a pie chart. Numbers indicate the percentage of institutions using these criteria.

Table 4. Definition of LSBE

Country	Definition of LSBE	
	circumferentially >3 cm	maximally >3 cm
China	33.3	66.7
Korea	20.0	80.0
Japan	73.3	26.7
Thailand	14.3	85.7
Indonesia	80.0	20.0
Philippines	0	100

We asked about the definition of long segment Barrett's esophagus (LSBE) used in each country, with the following presented as options: circumferentially greater than 3 cm, maximally greater than 3 cm, and others. Values shown indicate the percentage of institutions using the indicated definition.

BE is subdivided into LSBE and SSBE. Although this is an arbitrary distinction stemming from the origin of BE, it has important clinical relevance. Thus, we asked regarding the definition of LSBE. In Japan and Indonesia, a greater than 3 cm circumferential length is thought to be necessary to define LSBE, while in the other countries, a greater than 3 cm maximal length is considered to be sufficient (table 4). These findings reveal different opinions regarding the definition of LSBE among East Asian countries.

Epidemiology of BE and Barrett's Adenocarcinoma

The reported prevalence of BE in Asian counties is low as compared to Western countries. To clarify the current situation regarding prevalence of BE in East Asia, we asked about the prevalence of the disease in each institution. In about 80% of the responding institutions, fewer than 10 patients were diagnosed as having LSBE in a single year and the differences among the countries were not large (fig. 2a). Similarly, in over 90% of the institutions, fewer than 10 patients were newly diagnosed with LSBE in a single year (fig. 2b). Consistent with previously published data [5], the number of SSBE patients was much higher in Japan than in the other East Asian countries (fig. 3a, b).

Subsequently, we sought to clarify the current situation regarding the prevalence of Barrett's adenocarcinoma. In most of the institutions, fewer than 5 patients were diagnosed with Barrett's adenocarcinoma in a single year (fig. 4). Since more than 3,000 EGD examinations were performed in that year at over half of the institutions, the prevalence of Barrett's adenocarcinoma is considered to be extremely low in East Asia. In Asian countries, the majority of esophageal cancer cases are squamous cell carcinoma. Although the number of reports is few, some recent studies have shown the incidence of Barrett's adenocarcinoma in Asia is rising [6]. To elucidate the ratio of Barrett's adenocarcinoma among all cases of esophageal cancer in East Asia, we asked regarding the ratio of Barrett's adenocarcinoma among esophageal cancer cases. Table 5 shows the frequency of Barrett's adenocarcinoma among total cases of esophageal cancer. In China, Korea, Japan, and Thailand, the frequency of Barrett's adenocarcinoma is below 5% of all esophageal cancer cases. In contrast, in Indonesia and the Philippines, the percentage is more than 10% of total esophageal cancer cases at 80 and 38%, respectively, of the queried institutions in those countries.

Management of BE

Current guidelines from the ACG recommend endoscopic surveillance with four-quadrant biopsies to detect dysplastic lesions of BE, termed the 'Seattle biopsy protocol', as a more effective surveillance method has not been established [13, 23]. However, a number of limitations including sampling error, and time- and cost-effectiveness have been reported [24, 25]. To elucidate the current situation regarding surveillance programs for BE in East

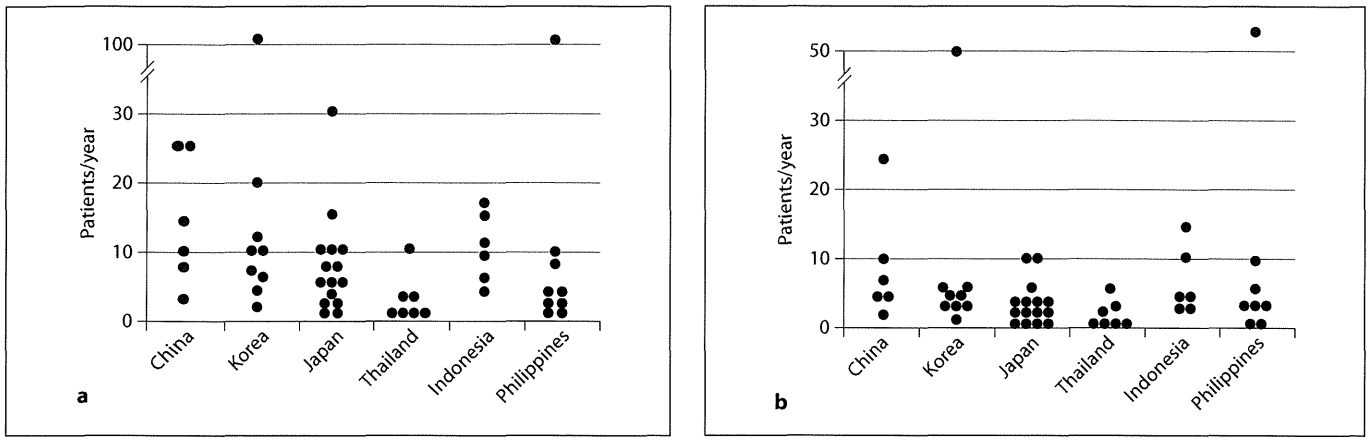

Fig. 2. Prevalence of LSBE in most recent year in each country. We asked about the prevalence of LSBE in the most recent year in each country and the findings are presented as a distribution chart. **a** The approximate number of cases diagnosed with LSBE in the most recent year are shown on the vertical axis, with each country placed on the horizontal axis. **b** Number of patients newly diagnosed with LSBE.

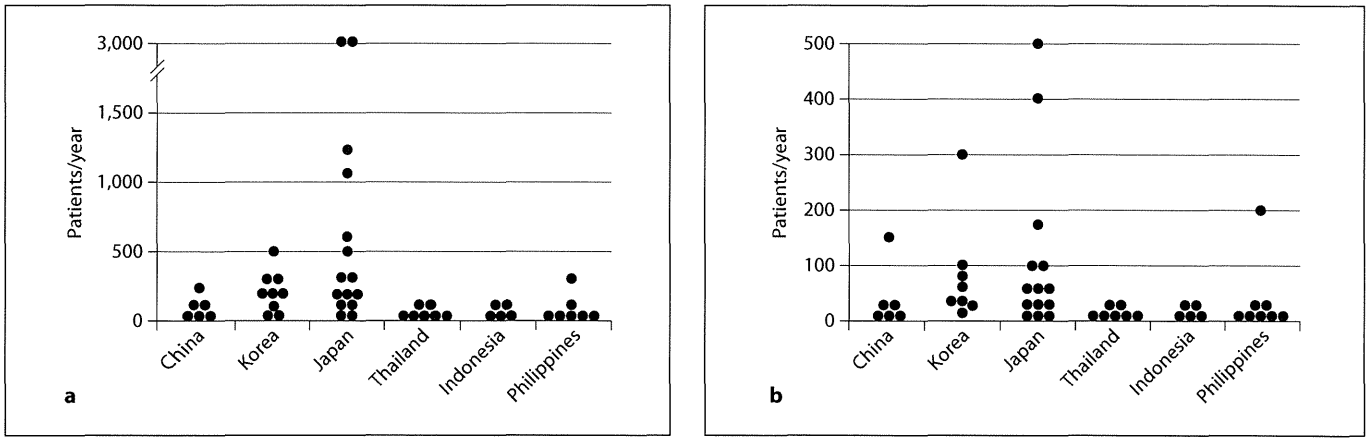

Fig. 3. Prevalence of SSBE in most recent year in each country. We asked about the prevalence of SSBE in the most recent year in each country and the findings are presented as a distribution chart. **a** The approximate numbers of cases diagnosed with SSBE in the most recent year are shown on the vertical axis, with each country placed on the horizontal axis. **b** Number of patients newly diagnosed with SSBE.

Asia, we asked about use of the Seattle biopsy protocol for endoscopic surveillance of BE. The answers showed that the protocol is utilized in around 30% of the institutions in most of the queried countries (fig. 5). However, none of the queried institutions in Japan use this protocol.

Currently, management of BE is focused on treating reflux and managing the risk of cancer development. Reflux control is achieved by acid suppression with proton pump inhibitors (PPIs) [26, 27] or surgery. Since epidemiology studies have shown that patients receiving chronic NSAID administration have about half the rate of esophageal cancer, as compared with the general public [28], NSAID use has been postulated to diminish the incidence of BE or at least delay its progression to cancer. Thus, we sought to elucidate the current situation regarding management of BE by use of these drugs. Except for 1 institution, PPIs are administered for patients with BE in all of the queried countries. In about half of the institutions, PPIs are administered for reflux symptoms, while they are administered for both reflux symptoms and pre-

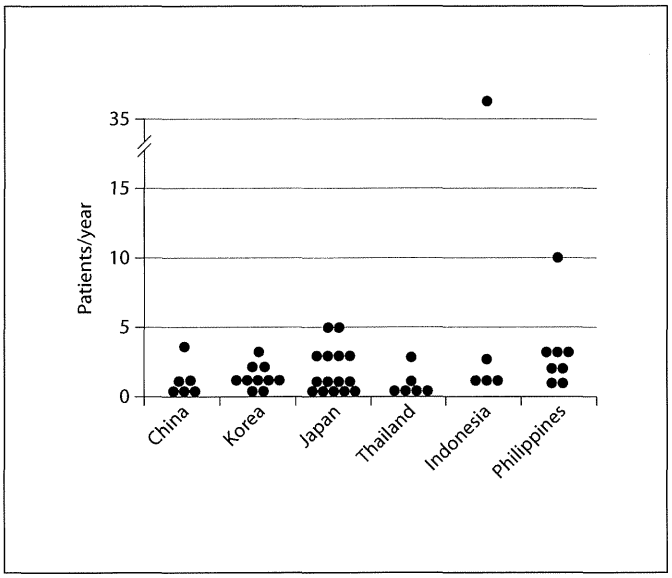

Fig. 4. Prevalence of Barrett's adenocarcinoma in most recent year in each country. We asked about the prevalence of Barrett's adenocarcinoma in the most recent year in each country and the findings are presented as a distribution chart. The approximate numbers of cases diagnosed with Barrett's adenocarcinoma in the most recent year are shown on the vertical axis, with each country placed on the horizontal axis.

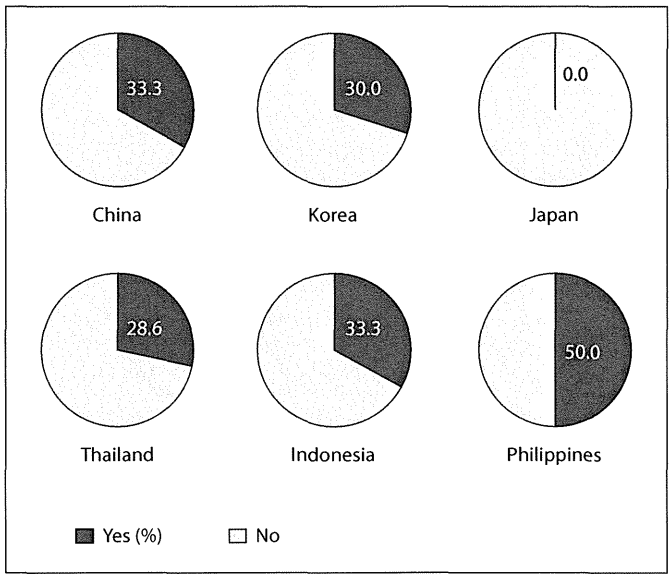

Fig. 5. Use of Seattle biopsy protocol for endoscopic surveillance of Barrett's esophagus. We asked about the use of the Seattle biopsy protocol for endoscopic surveillance of Barrett's esophagus in each country and the answers are presented as a pie chart. Numbers indicate the percentage of institutions using this protocol.

Table 5. Frequency of Barrett's adenocarcinoma

Country	Frequency of Barrett's adenocarcinoma among total esophageal carcinoma cases			
	<1%	1–5%	5–10%	>10%
China	3 (60)	2 (40)	0	0
Korea	8 (80)	2 (20)	0	0
Japan	8 (53)	5 (33)	2 (13)	0
Thailand	3 (60)	2 (40)	0	0
Indonesia	1 (20)	0	0	4 (80)
Philippines	1 (13)	4 (50)	0	3 (38)

Values shown indicate the number (%) of institutions in each country.

venting dysplastic progression in the other half (online suppl. fig. S1; for all online supplementary material, see www.karger.com/doi/10.1159/000339778). In contrast to PPIs, NSAIDs are not administered for patients with BE in most of the surveyed institutions, except for 2. In regard to PPIs and NSAIDs for patients with BE, there were no significant differences among the countries.

Advanced Endoscopic Imaging for Diagnosis of BE

Next, we enquired about the availability and usefulness of IEE procedures for diagnosis of Barrett's adenocarcinoma in each country. Although NBI is widely used in most Asian countries, other modalities are not (table 6). Moreover, NBI is thought to be the most useful modality for such a diagnosis among IEE procedures, while chromoendoscopy is also thought to be useful (table 7). On the other hand, AFI and acetate-enhanced endoscopy are thought to be less useful than the other modalities.

Treatment of Dysplastic Lesions of BE

Finally, we asked about current opinions regarding treatment of high-grade dysplasia and mucosal carcinoma in BE in each country. As shown in figure 6, endoscopic treatment is well accepted for high-grade dysplasia and mucosal carcinoma in BE in all of the surveyed countries. Then, we asked an additional question about the indication of endoscopic treatment for dysplastic lesions in BE. Although many of the endoscopists left this answer blank, intramucosal cancer is thought to be an indication for endoscopic treatment in 30 to 70% of the institutions, except for those in Indonesia (online suppl.

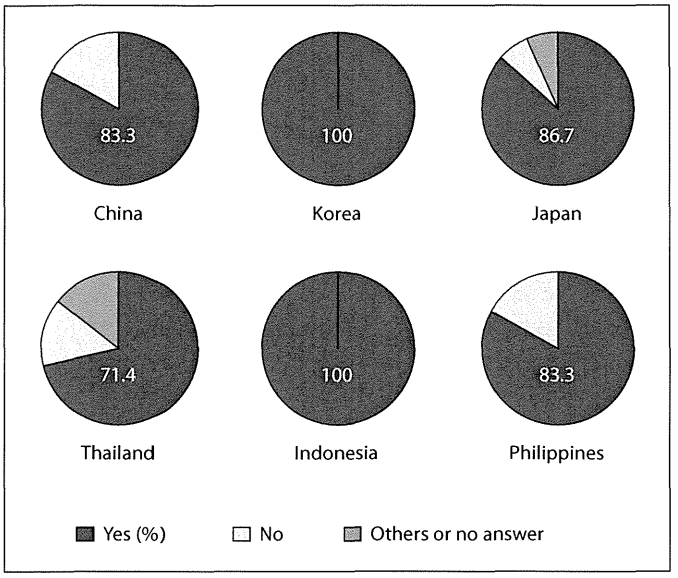

Fig. 6. Indication of endoscopic treatment for high grade dysplasia and mucosal carcinoma related to Barrett's esophagus. We asked whether the endoscopists in each country agree that endoscopic treatment is suitable for high-grade dysplasia and mucosal carcinoma related to Barrett's esophagus and the answers are presented as a pie chart for each country. Numbers indicate the percentage of institutions who consider endoscopic treatment acceptable for high-grade dysplasia and mucosal carcinoma related to Barrett's esophagus.

Table 6. Experience with usage of advanced endoscopic imaging for diagnosis of Barrett's esophagus

Country	Endoscopic modalities			
	NBI	AFI	acetate enhanced	chromo-endoscopy
China	33.3	0	16.7	50.0
Korea	80.0	30.0	10.0	20.0
Japan	80.0	0	20.0	26.7
Thailand	57.1	0	0	28.6
Indonesia	100	16.7	16.7	83.3
Philippines	91.7	0	0	36.4

We asked about experience with usage of advanced endoscopic imaging for diagnosis of Barrett's esophagus in each country. Values shown indicate the percentage of institutions using the indicated modality. NBI = Narrow band imaging; AFI = autofluorescence imaging.

Table 7. Usefulness of advanced endoscopic imaging for diagnosis of Barrett's adenocarcinoma

Country	Endoscopic modalities			
	NBI	AFI	acetate enhanced	chromo-endoscopy
China	83.3	33.3	50.0	66.7
Korea	60.0	30.0	10.0	20.0
Japan	66.7	20.0	20.0	46.7
Thailand	71.4	0	28.6	85.7
Indonesia	100	50.0	80.0	100
Philippines	83.3	25.0	36.4	72.7

We asked regarding opinions about the effectiveness of each endoscopic modality for diagnosis of Barrett's adenocarcinoma in each country. Values shown indicate the percentage of institutions that consider that the indicated modality is effective for diagnosis of Barrett's adenocarcinoma. NBI = Narrow band imaging; AFI = autofluorescence imaging.

fig. S2). We also asked about the appropriate treatment modality for endoscopic treatment of dysplastic lesions of BE and found that modalities considered to be appropriate differ among the countries. ESD is thought to be the most appropriate in Korea, and Japan, while RFA is considered to be more appropriate in Thailand (online suppl. fig. S3).

Discussion

This is the first multinational investigation of East Asian countries to address various aspects of BE. Here, we intend to provide a point-by-point discussion of the current diagnosis, epidemiology, management, and treatment of BE based on responses from major institutions in East Asian countries to a questionnaire-based survey.

The definitions of BE vary widely among different countries. For example, in the United States, BE is defined as metaplastic replacement of any length of the esophageal epithelium that is confirmed to have specialized intestinal metaplasia in biopsy findings [13]. On the other hand, in the United Kingdom [29] and Japan [30], BE is defined simply as columnar lined esophagus with or without intestinal metaplasia. Thus, one of the most important issues regarding diagnosis of BE is whether the presence of intestinal metaplasia is required. In the present survey, the presence of SCE is considered to be important for diagnosing BE in East Asian countries, except for Japan. The Japan Esophageal Society defines BE as having at least one of the following pathological findings: (1)

esophageal glands or ducts beneath the overlying columnar epithelium; (2) squamous epithelial islands located in the columnar epithelium, and (3) double layers of muscularis mucosa beneath the overlying columnar epithelium [30]. Thus, Japanese endoscopists may attach less importance to the presence of SCE. Notably, the presence of squamous islands in columnar epithelium confirmed by endoscopy was accepted for diagnosis of BE in all of the surveyed countries. Recently, we reported that endoscopic identification of squamous islands by NBI was helpful to improve diagnostic concordance of SSBE [31]. Endoscopic diagnosis of SSBE by identification of squamous islands in columnar mucosa may also be beneficial because it can reduce the risk of complications, such as bleeding.

According to a validation study, the C&M criteria have a high overall validity for endoscopic assessment of visualized BE length [32]. However, the co-efficient was less valid in that study in cases with columnar epithelial lining less than 1 cm. Low diagnostic concordance was also consistently found for BE with a length of less than 1 cm among Asian endoscopists [33]. These findings complicate the universal standardization of endoscopic diagnosis of BE, because most cases of BE in Asian countries are less than 1 cm in length. Not surprisingly, awareness of the C&M criteria for endoscopic diagnosis of BE was shown to be inadequate in East Asian countries. In Japan, the distal end of the esophageal palisade vessels is frequently used as a landmark for the EGJ and has been proposed by the Japan Esophageal Society [30]. Therefore, Japanese endoscopists prefer to use the palisade vessels to define the EGJ. In contrast, most of the institutions queried use both landmarks for the definition of EGJ. While, in the case of SSBE, the ratio of using the esophageal palisade vessels as a primary landmark for EGJ are increased nearly a third of institutions (data not shown). In addition to the low diagnostic concordance in SSBE, the proximal end of gastric folds is frequently unable to recognize in patients with severe atrophic gastritis by *Helicobacter pylori* infection which is well known to be more prevalent in Asian than in Western countries. Therefore, the distal end of palisade vessels may be easier to identify EGJ in the case of SSBE with *H. pylori* infection, although it remains controversial which landmark to use for the endoscopic diagnosis of SSBE [9, 10]. A new modification of the C&M criteria may be necessary for more accurate and suitable use in patients with SSBE.

Consistent with previously published findings, LSBE prevalence is extremely low in East Asia, while that of SSBE is very high only in Japan. These differences may be caused by different definitions used for the EGJ and BE. In contrast, the survey results indicated that the incidence of Barrett's adenocarcinoma may be rising in Indonesia and the Philippines. Moreover, the ratio of Barrett's adenocarcinoma among all cases of esophageal cancer has increased to over 10% in Indonesia and the Philippines, while that remains below 5% in the other surveyed countries. Although the precise rate of incidence in each of the surveyed countries remains vague, these differences may be caused by genetic or racial differences as well as lifestyle factors such as abdominal adiposity.

Our findings indicate that most endoscopists in East Asian countries do not follow the endoscopic surveillance program including four quadrant biopsies every 2 cm of the BE segment (Seattle protocol) as recommended by the American gastroenterology society [13, 34]. Notably, none of the institutions in Japan conduct this protocol. Adherence to the Seattle protocol has been reported to be insufficient also in Western countries. An American study using a national community-based pathology data base, adherence to Seattle protocol was found only 51% [35], which was slightly higher than that in this study. Consistently, according to several survey studies, only 41–77% of endoscopists adhere to the protocol in clinical practice [36–38]. These data indicate that endoscopists in Western countries often do not follow the recommended biopsy protocol, which is labor-intensive and tedious. Moreover, repeated biopsies can result in scars in the esophageal mucosa and hamper endoscopic therapy, such as ESD. Therefore, techniques to improve the efficacy of screening and surveillance strategies are highly desirable. Recently, international, randomized, crossover trial comparing white light endoscopy using Seattle protocol and NBI with targeted biopsies was conducted [14]. The results of this study showed that NBI with targeted biopsies could have the same detection rate of intestinal metaplasia as white light endoscopy using Seattle protocol, while fewer biopsies. In addition, NBI with targeted biopsies can detect more areas with dysplasia. Collectively, NBI with targeted biopsies may be a new standard protocol to improve the efficiency of current endoscopic screening and surveillance practice in patients with BE and also reduce costs.

The present survey findings show that NBI is now widely available in East Asian countries and this modality is thought to be most useful for detection of Barrett's adenocarcinoma among the queried endoscopists. Although some endoscopic classifications have been proposed for NBI findings [39, 40], they are too complicated to become universally standardized and a simpler clas-

sification is necessary for effective surveillance of BE, especially for less experienced endoscopists.

In this survey, most endoscopists in East Asia accepted endoscopic treatment for high-grade dysplasia and mucosal cancer related to BE. However, available techniques vary widely among countries. As compared to Western countries, endoscopists in East Asia, especially Korea and Japan, tend to avoid ablation therapies, such as RFA, which are not able to histologically assess the depth of dysplastic lesion and effectiveness of the therapy. Although endoscopists currently have a variety of techniques to choose from when treating BE with dysplasia, there are numerous issues that remain to be solved. Indeed, most of the concerns for the endoscopic management were related to the endoscopic therapy for dysplastic lesions of BE, including appropriate indication, evaluation of depth of invasion, selection of the endoscopic procedure, and management after therapy (response to Q33). Standardization of the various classification systems as well as incorporation of techniques into a simply managed unit that is cost-effective and less time-consuming should eventually lead to widespread availability in East Asian countries.

There are some limitations in this study. First, the number of participating institutions may not be large enough to reflect the major opinions in each country. Moreover, there were differences in the number of participating institutions among surveyed countries. Therefore, selection bias may affect the present results. Second, responses to the questionnaire were collected by institution, not by endoscopists. However, each endoscopist in the same institution may have different opinion regarding endoscopic management of Barrett's esophagus. Third, the present study relied on a questionnaire-based survey answered by institutions, so the data are not representative of the patient's perspective precisely, particularly with the respect to the epidemiology of BE and Barrett's adenocarcinoma. These limitations necessitate future studies to validate, although the present study gives important information for understanding the opinion regarding the management of BE in each country.

In conclusion, we attempted to clarify differences among institutions in East Asian countries in regard to endoscopic management of BE and Barrett's adenocarcinoma. Among the countries queried, there were both similarities and differences regarding diagnosis and management of BE, with the different opinions regarding diagnosis of BE between Japan and other East Asian countries notable. This survey reveals important information about the current situation as well as problems related to endoscopic management in East Asian countries. However, a number of unresolved issues in management of BE remain and further investigation is needed to determine the best strategy for affected patients in East Asia.

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Appendix

Questionnaire used in the present survey	
Q1	Please describe your country.
Q2	How many patients are investigated by esophago-gastro-duodenoscopy (EGD) in a recent year at your institution? _____ patient/year
Q3	Do you use the following criteria for the diagnosis of Barrett's esophagus? 1. Specialized intestinal metaplasia (biopsy) Yes or No 2. Squamous island in columnar epithelium (endoscopy) Yes or No 3. Esophageal proper gland (histology) Yes or No 4. Double layer of muscularis mucosae (histology) Yes or No
Q4	Do you use C&M criteria for the endoscopic diagnosis of Barrett's esophagus? Yes or No or I do not know C&M criteria
Q5	How do you define long segment Barrett's esophagus (LSBE)? > circumferentially 3 cm or > maximally 3 cm or others (describe your definition)
Q6	How many LSBE patients do you diagnose in a recent year at your institution? _____ patient/year
Q7	How many new LSBE patients do you diagnose in a recent year at your institution? _____ patient/year
Q8	How many SSBE patients do you diagnose in a recent year at your institution? _____ patient/year
Q9	How many new SSBE patients do you diagnose in a recent year at your institution? _____ patient/year
Q10	Do you think that LSBE is increasing at your institution? Yes or No
Q11	Do you think that SSBE is increasing at your institution? Yes or No
Q12	How many Barrett's adenocarcinoma do you diagnose in a recent year at your institution? Yes or No
Q13	What percent of total esophageal carcinoma is Barrett's adenocarcinoma at your institution? _____ %
Q14	Do you think that Barrett's adenocarcinoma is increasing at your institution? Yes or No or I do not know
Q15	What kind of the endoscopic landmark do you use to identify the esophago-gastric junction (EGJ)? The upper end of gastric folds or the lower end of esophageal palisade vessels or both or neither (describe your landmark)

Q16	If your answer 'Both' in Q15: What kind of the endoscopic landmark do you use to identify the EGJ in case with LSBE? The upper end of gastric folds or the lower end of esophageal palisade vessels or both or neither (describe your landmark) What kind of the endoscopic landmark do you use to identify the EGJ in case with SSBE? The upper end of gastric folds or the lower end of esophageal palisade vessels or both or neither (describe your landmark)
Q17	Is the endoscopic surveillance for Barrett's esophagus by Seattle biopsy protocol performed at your institution? Yes or No
Q18	Do you think that specialized columnar epithelium is important in the surveillance? Yes or No
Q19	Do you think that specialized columnar epithelium is important as a marker of highly risky precancerous lesion of Barrett's adenocarcinoma? Yes or No
Q20	Do you administer PPI for patients with Barrett's esophagus? No Yes, I administer PPI for reflux symptoms Yes, I administer PPI for preventing dysplastic progression Yes, I administer PPI for preventing dysplastic progression, only when patients have dysplastic Barrett's esophagus Yes, I administer PPI both for reflux symptoms and for preventing dysplastic progression Yes, for other reasons (describe your reason of PPI administration)
Q21	Do you administer aspirin/NSAIDs to patients with Barrett's esophagus? No Yes, I administer aspirin/NSAIDs for preventing dysplastic progression Yes, I administer aspirin/NSAIDs for preventing dysplastic progression, only when patients have dysplastic Barrett's esophagus Yes, for other reasons (describe your reason of aspirin/NSAIDs administration)
Q22	Have you used narrow band image (NBI) endoscopy for the diagnosis of Barrett's esophagus? Yes or No
Q23	Do you think that NBI endoscopy is useful for the diagnosis of Barrett's carcinoma? Yes or No or I do not know
Q24	Have you used autofluorescence image (AFI) endoscopy for the diagnosis of Barrett's esophagus? Yes or No
Q25	Do you think AFI endoscopy is useful for the diagnosis of Barrett's carcinoma? Yes or No or I do not know
Q26	Have you used acetate-enhanced endoscopy for the diagnosis of Barrett's esophagus? Yes or No
Q27	Do you think that acetate-enhanced endoscopy is useful for the diagnosis of Barrett's carcinoma? Yes or No or I do not know
Q28	Have you used chromoendoscopy for the diagnosis of Barrett's esophagus? Yes or No
Q29	Do you think that chromoendoscopy is useful for the diagnosis of Barrett's carcinoma? Yes or No or I do not know
Q30	Do you agree that the endoscopic treatment is suitable for high-grade dysplasia and mucosal carcinoma of Barrett's esophagus? Yes or No or others (describe reason)
Q31	If you answer 'Yes' in Q30: What is your indication for the endoscopic treatment?
Q32	If you answer 'Yes' in Q30: Which endoscopic treatment is considered to be the most appropriate one? Endoscopic mucosal resection (EMR) Endoscopic submucosal dissection (ESD) Radiofrequency ablation Cryo ablation Photodynamic therapy Electrocoagulation Argon plasma ablation Others (describe)
Q33	Are there any concerns for the endoscopic management of Barrett's esophagus and/or Barrett's adenocarcinoma?

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Low-density Lipoprotein Receptor-related Protein-1 (LRP1) Mediates Autophagy and Apoptosis Caused by *Helicobacter pylori* VacA*[S]

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Background: *Helicobacter pylori* VacA receptor(s) responsible for apoptotic cell death and autophagy has not been identified.
Results: VacA-induced autophagy via low-density lipoprotein receptor-related protein-1 (LRP-1) binding precedes apoptosis.
Conclusion: LRP1 mediates VacA-induced autophagy and apoptosis.
Significance: This study identified LRP1 as a VacA receptor associated with toxin-induced autophagy and apoptosis and demonstrated its importance in the processes.

In *Helicobacter pylori* infection, vacuolating cytotoxin (VacA)-induced mitochondrial damage leading to apoptosis is believed to be a major cause of cell death. It has also been proposed that VacA-induced autophagy serves as a host mechanism to limit toxin-induced cellular damage. Apoptosis and autophagy are two dynamic and opposing processes that must be balanced to regulate cell death and survival. Here we identify the low-density lipoprotein receptor-related protein-1 (LRP1) as the VacA receptor for toxin-induced autophagy in the gastric epithelial cell line AZ-521, and show that VacA internalization through binding to LRP1 regulates the autophagic process including generation of LC3-II from LC3-I, which is involved in formation of autophagosomes and autolysosomes. Knockdown of LRP1 and *Atg5* inhibited generation of LC3-II as well as cleavage of PARP, a marker of apoptosis, in response to VacA, whereas caspase inhibitor, benzyloxycarbonyl-VAD-fluoromethylketone (Z-VAD-fmk), and necroptosis inhibitor, Necrostatin-1, did not inhibit VacA-induced autophagy, suggesting that VacA-induced autophagy via LRP1 binding precedes apoptosis. Other VacA receptors such as RPTPα, RPTPβ, and fibronectin did not affect VacA-induced autophagy or apoptosis. Therefore, we propose that the cell surface receptor, LRP1, mediates VacA-induced autophagy and apoptosis.

Helicobacter pylori colonizes more than half the world's population. Although persistent infection by *H. pylori* is accepted as a major cause of gastroduodenal diseases (*e.g.* peptic ulcer disease, gastric lymphoma, gastric adenocarcinoma), the responsible cellular pathways have not been defined. Variation in manifestations of *H. pylori* infection in different populations suggests differences in virulence of strains, host genetic susceptibility, and responses to environmental factors. Many *H. pylori* strains isolated from patients contain the *cagA* gene (cytotoxin-associated gene A) as well as produce the vacuolating cytotoxin, VacA. Additional *H. pylori* products, including urease, OipA, adhesins, heat-shock protein, and lipopolysaccharide appear to be involved in virulence (1, 2).

Interestingly, VacA causes epithelial damage in mouse models both when given orally as a single agent (3) and when delivered by a toxigenic strain of *H. pylori* during gastric infection (4, 5). *In vitro*, VacA is internalized by endocytosis (6), which is inhibited by CagA (7, 8), and exerts multiple effects on susceptible cells, including vacuolation and mitochondrial damage, leading eventually to apoptosis (9–13). In addition, VacA forms hexametric pores, followed by endocytosis and processing into late-endosomal compartments (14), which then undergo osmotic swelling to become large acidic vacuoles. Although vacuolation is the most obvious effect of VacA *in vitro*, it is not as obvious *in vivo*. The pleiotropic effects of VacA appear to result from activation of different signal transduction pathways through binding to several epithelial cell receptors, *e.g.* receptor protein-tyrosine phosphatase (RPTP)[3] β and α (15, 16), fibronectin (FN) (17), sphingomyelin (18).

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[3] The abbreviations used are: RPTP, receptor protein-tyrosine phosphatase; LRP1, low-density lipoprotein receptor-related protein; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; DIDS, 4,4′-diisothiocyanostibene-2,2′-disulfonic acid; MAA, *Maackia amurensis*; FN, fibronectin; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; PARP, poly(ADP-ribose) polymerase.

VacA enhanced tyrosine phosphorylation of the G protein-coupled receptor kinase-interactor 1 (Git1) as did pleiotrophin, an endogenous ligand of RPTPβ (19). Oral administration of VacA to wild-type mice, but not to *RPTPβ* knock-out mice, resulted in gastric ulcer. However, cells lacking RPTPβ were able to internalize VacA and undergo vacuolation (20), suggesting that other VacA receptors were responsible for vacuolation. Recent interest has focused on the immunosuppressive effects of VacA, *i.e.* VacA inhibited proliferation of T cells due to down-regulation of interleukin-2 (IL-2) transcription (21, 22). Through interactions with the β2-integrin subunit CD18 of the leukocyte-specific integrin LFA-1 (23), VacA plays an important role in inhibition of interleukin-2 (IL-2) gene expression after clathrin-independent endocytosis via PKC-dependent phosphorylation of the cytoplasmic tail of CD18 (24). Thus, VacA has effects on both epithelial cells (25) as well as inflammatory cells (26).

Over the last 10 years, studies have focused on the mechanism of cell death resulting from mitochondrial damage caused by VacA (10, 12, 13, 27). Additional recent studies have shown that VacA induces autophagy, but the pathway has not been identified (28, 29). Autophagy can promote the survival of dying cells (30). However, increased autophagic activity can also lead to cell death (31–35), suggesting that autophagy can be responsible for both cytoprotective and cytotoxic activities, depending on the specific cellular conditions.

Here we purified from AZ-521 cells, a human gastric epithelial cell line, a surface membrane protein, p500, which binds VacA, and identified it as low-density lipoprotein receptor-related protein-1 (LRP1). LRP1 binding of VacA was shown to be specifically responsible for VacA-induced autophagy and apoptosis. Similar to RPTPα and RPTPβ, LRP1 mediates VacA internalization in AZ-521 cells, but in contrast to RPTPα and RPTPβ, LRP1 targeted downstream pathways leading to autophagy and apoptosis.

EXPERIMENTAL PROCEDURES

Antibodies and Other Reagents—Anti-LC3B, anti-cleaved caspase-7, anti-cleaved PARP, anti-Beclin-1, and anti-mammalian target of rapamycin antibodies were from Cell Signaling. Mouse monoclonal antibodies reactive with LRP1 (8G1) were from Santa Cruz Biotechnologies; those reactive with RPTPβ were from BD Biosciences; and those reactive with LC3 (clone 1703) were from Cosmo Bio. Anti-RPTPβ antibody was raised against its extracellular domain, corresponding to the N-terminal amino acids of the human protein (36). Anti-RPTPα rabbit polyclonal antibodies for immunoblotting were provided by Dr. Jan Sap and anti-RPTPα rabbit polyclonal antibodies for immunofluorescence experiments were raised against its extracellular domain, corresponding to the N-terminal amino acids of the human protein; mouse monoclonal antibodies reactive with α-tubulin, necrostatin-1, and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) were from Sigma. Diamidino-2-phenylindole dihydrochloride (DAPI) and 4,4′-diisothiocyanostibene-2,2′-disulfonic acid (DIDS) were from Invitrogen. A general caspase inhibitor, Z-VAD-fmk was from BD Pharmingen. 3-Methyladenine was from MP Biomedicals.

Cell Culture and Gene Silencing—AZ-521 cells, a human gastric cancer cell line obtained from the Japan Health Sciences Foundation, were cultured in Earle's minimal essential medium (Sigma) containing 10% fetal calf serum. AGS cells, a human gastric cancer cell line, were cultured in RPMI1640 (Sigma) containing 10% fetal calf serum. Cells were plated into 24-well dishes (5×10^4 cells/well) or 12-well dishes (1×10^5 cells/well) in Earle's minimal essential medium containing 10% FCS. RNA interference-mediated gene knockdown was performed using validated Qiagen HP small-interfering RNAs (siRNAs) for mammalian target of rapamycin (SI00300244). The validated LRP1 siRNA was purchased from Ambion. Beclin-1 siRNA was designed and validated as described by Høyer-Hansen *et al.* (37). Atg5 siRNAs (Atg5-1, agugaacaucugagcuacccggaua; Atg5-2, caaucccauccagaguugcuuguga) were designed and validated as described by Yang *et al.* (38). RPTPβ siRNA (5′-gcacaagaaucgauaacaua-3′) and RPTPα siRNA (5′-cgaagagaauacagacuau-3′) were synthesized by B-Bridge. Negative-control siRNAs were purchased from Sigma. AZ-521 cells were transfected with 100 nM of the indicated siRNAs for 48–72 h using Lipofectamine™ RNAiMax transfection reagent (Invitrogen) according to the manufacturer's protocol. Knockdown of the target proteins was confirmed by immunoblotting with the indicated antibodies.

RPTPα shRNA Expression Vector Construction and Transfection—The three highest scoring shRNA sequences targeted for human RPTPα were chosen by B-Bridge International, Inc.: RPTPα siRNA1, 5′-cggcagaaccagttaaaga-3′; RPTPα siRNA2, 5′-gcaccaacattcagcccaa-3′; RPTPα siRNA3, 5′-ggagaatggcagacgacaa-3′. The shRNA negative control, obtained from B-Bridge International, Inc. (Tokyo, Japan), has no homology to any human mRNA sequences in the NCBI Reference Sequence Database. We used the pSH1-H1-H1-Puro shRNA Lentiviral Expression System (SBI Inc.) to generate lentivirus supernatants from HEK293FT cells. In brief, HEK293FT cells were seeded in 10-cm dishes at 5×10^6 cells/dish. After cells reached 90–95% confluence, the constructed shRNA expression vector (3 μg/dish) in ViraPower Packaging Mix (9 μg/dish) with Lipofectamine 2000 (Invitrogen Inc.) was transfected into HEK293FT cells. Twelve hours after initiating transfection, the plasmid/Lipofectamine solution was removed, and cell growth medium without antibiotics was added. The lentivirus-containing supernatants were harvested 48 and 72 h post-transfection. The AZ-521 cells were plated to 30–50% confluence and transfected with appropriate dilutions of lentivirus supernatants. 24 h after transfection, the cells were cultured in cell growth medium containing puromycin (0.5 μg/ml) to obtain the stable, transfected AZ-521 cells. After several selections, we isolated AZ-521 cells with knockdown of endogenous RPTPα.

Purification of VacA—The toxin-producing *H. pylori* strain ATCC 49503 was the source of VacA for purification as previously described (36).

Assay for Vacuolating Activity—Vacuolating activity was assessed using AZ-521 cells as previously described (36). Briefly, cells (1×10^4 cells/well, 100 μl) were grown as monolayers in 96-well culture plates for 24 h in a 5% CO_2 atmosphere at 37 °C. VacA was added, and cells were incubated at 37 °C for

the indicated times. To quantify vacuolating activity, the uptake of neutral red into vacuoles was determined.

Preparation of Alexa 555-labeled VacA—To investigate VacA binding to cells and co-localization with other proteins in cells, VacA was labeled using the Alexa Fluor 555 Protein Labeling Kit (Molecular Probes), according to instructions provided by the manufacturer. In brief, 50 μl of 1 M sodium bicarbonate buffer (pH 8.5) were added to 500 μl (500 μg in phosphate-buffered saline (PBS)) of VacA, followed by incubation with the reactive dye in the vial for 15 min at room temperature. To remove excess dye, the reaction mixture was applied to a PD-10 column (Amersham Biosciences). Alexa 555-labeled VacA (100 μg/ml) was stored at −20 °C.

Purification and Identification of p500—To purify p500 using affinity columns, AZ521 cells (5×10^7 cells) were washed twice with PBS, and suspended in 10 ml of Sol buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 1% Triton X-100, with protease inhibitor mixture (Roche Diagnostics)) for 15 min on ice. After centrifugation (20 min at 17,400 × *g*), the supernatant was filtered (0.45 μm, Millipore) and the filtrate (10 ml) applied to a *Maackia amurensis* (MAA)-agarose column (2 ml bed volume, Seikagaku Corporation). After washing the column, Sol buffer containing 50 mM ethylenediamine was used to elute the carbohydrate-containing proteins in 1-ml fractions. To confirm the presence of p500 in the eluted fractions, proteins in effluents were detected by lectin blotting using MAA as described previously (15, 16). To identify p500, proteins in effluents were precipitated with chloroform/methanol, then heated at 100 °C for 10 min in 1× SDS-PAGE sample buffer, separated in 6% gels, and transferred to PVDF membranes, which were stained with Coomassie Brilliant Blue. The stained bands were used for LC-MS/MS analysis.

Immunoprecipitation—Immunoprecipitation of VacA-binding proteins from AZ521 cells was performed as described previously. In brief, biotinylated AZ521 cell lysates (100 μg/200 μl) were incubated at 4 °C for 1 h with 1 μg of native VacA or heat-inactivated VacA (100 °C, 10 min), followed by incubation overnight at 4 °C with 1 μl of rabbit anti-VacA antibodies. Antibody-bound proteins were collected after addition of 20 μl of rProtein G-agarose (Invitrogen), 50% (v/v) in Sol buffer, and incubated at 4 °C for 1.5 h. After the beads were washed three times with Sol buffer, proteins were solubilized in SDS-PAGE sample buffer, resolved by SDS-PAGE, and transferred to PVDF membranes (Millipore; Immobilon-P membranes), which were incubated with streptavidin-HRP (Amersham Biosciences). Biotinylated proteins were detected using the enhanced chemiluminescence system (Pierce).

Immunofluorescence Confocal Microscopy—For immunofluorescence analysis of VacA co-localization with LRP1, RPTPα, RPTPβ, or LC3B, AZ-521 cells (1×10^5 cells) on coverglass (Matsunami) were incubated with 120 nM Alexa 555-labeled VacA for the indicated times, cells were fixed with 4% paraformaldehyde (PFA) at room temperature for 15 min, washed with PBS twice, and then immediately permeabilized with ice-cold 100% methanol for 10 min at −20 °C. The cells are then rinsed three times with PBS and incubated with blocking buffer (5% goat serum, 0.3% Triton X-100 in PBS) at room temperature for 1 h. To visualize LRP1 (8G1 antibody, 1:50), RPTPα (antibody provided by Jan Sap, 1:100), RPTPβ (polyclonal, 1:250), or LC3B (D11, 1:200), cells were further incubated with the primary antibodies in 1% BSA/PBS buffer overnight at 4 °C, washed twice with PBS and incubated with anti-rabbit Alexa 488 (Molecular Probes), anti-mouse 488 (Molecular Probes), or anti-mouse Cy5 (Jackson ImmunoResearch Laboratories Inc.) antibodies at room temperature for 1 h in the dark. After washing with PBS three times, cells were mounted on glass slides using Prolong Gold Antifade reagent with DAPI. For staining the lysosomal compartment in VacA-treated cells, cells were incubated with 100 nM LysoTracker Red DND-99 (Molecular Probes) according to the instruction manual, before fixation with 4% paraformaldehyde. Colocalization of VacA and the indicated proteins was analyzed by FV10i-LIV confocal microscopy (Olympus). The images were arranged with Adobe Photoshop CS4.

Statistics—Densitometric analysis on the immunoblots was done by Image Gauge software (FUJI FILM). The *p* values for densitometric analysis and vacuolating assay were determined by Student's *t* test with GraphPad Prism software (GraphPad, San Diego, CA). *p* values of <0.05 were considered statistically significant.

RESULTS

Purification and Identification of p500—Our analysis of membrane proteins that bind VacA revealed three proteins, *i.e.* RPTPα, RPTPβ, and an unidentified p500. The latter protein had a molecular mass higher than RPTPβ and reacted with MAA lectin (15, 16). In the present study, we purified p500 using MAA-agarose column chromatography and identified it by LC-MS/MS as LRP1 (Fig. 1). We confirmed its association with native VacA by immunoprecipitation (Fig. 1).

LRP1 Mediates VacA Binding and Internalization in AZ-521 Cells—Confocal microscopy analysis revealed that in AZ-521 cells VacA colocalized with LRP1 on cell membranes, and was internalized, whereas heat-inactivated VacA did not show colocalization and internalization with LRP1 (data not shown) (Fig. 2*A*). Furthermore, AZ-521 cells transfected with siRNA of LRP1 did not show significant toxin binding resulting in internalization, suggesting that LRP1 mediates VacA binding to the cell surface and facilitates its internalization. In agreement with these data, silencing of the p500 gene inhibited vacuole formation caused by VacA (Fig. 2*B*). These results suggest that LRP1 is associated with toxin internalization.

VacA Induced Generation of LC3-II in an LRP1-dependent Manner—Based on the prior reports (28, 29) that VacA induced autophagy in AGS cells, we determined whether VacA induced LC3-II generation from LC3-I in AZ-521 cells. Consistent with previous findings, Western blot analysis showed that VacA induced LC3-II generation from LC3-I in a time-dependent manner (Fig. 3*a*). As expected, immunoblots of VacA-treated cells transfected with control siRNA indicated a progressive conversion over 10 h of LC3-I to LC3-II. In LRP1 siRNA-transfected cells, LRP1 expression was down-regulated after 4 h with VacA and conversion of LC3-II from LC3-I was suppressed (Fig. 3*b* and supplemental Fig. S1). These data suggest an important role of LRP1 in mediating autophagy in AZ-521 cells in response to VacA.

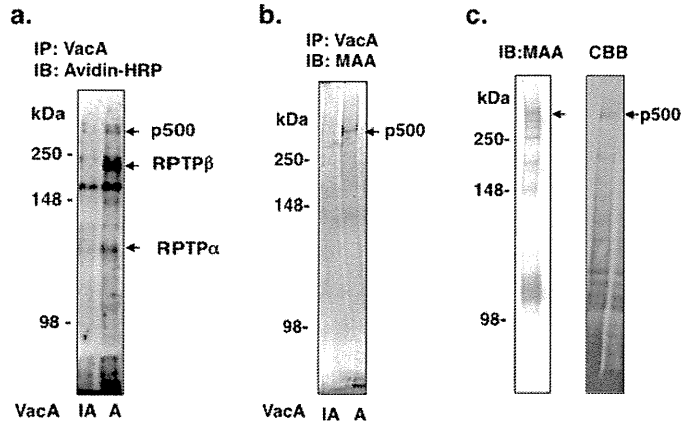


FIGURE 1. **Purification of p500 from AZ-521 cells by MAA-agarose column.** *a*, after biotinylation of surface proteins, AZ-521 cells were solubilized and immunoprecipitated with heat-inactivated (*IA*) or wild-type VacA (*A*) as described under "Experimental Procedures." Immunocomplexes were separated by SDS-PAGE in 6% gels and transferred to PVDF membranes. VacA-binding proteins were detected with streptavidin-HRP. *b*, proteins immunoprecipitated (*IP*) with heat-inactivated or wild-type VacA were separated by SDS-PAGE in 6% gels and transferred to PVDF membranes, which were incubated with MAA-lectin conjugated to digoxigenin and then with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase, followed by reaction with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate. *c*, biotinylated AZ-521 cell lysates were incubated overnight with a MAA-agarose column (2 ml bed volume), which was washed with 20 ml of Sol buffer. Bound proteins were eluted, concentrated, and separated by SDS-PAGE as described under "Experimental Procedures." MAA-lectin blotting is shown in the *left panel* and Coomassie Brilliant Blue (*CBB*) staining in the *right panel*. The stained p500 protein band was hydrolyzed with trypsin and subjected to LC-MS/MS analysis. The procedures described in *a*-*c* were repeated at least three times with similar results. *IB*, immunoblot.

VacA Induced Formation of Autophagosomes and Autolysosomes in AZ-521 Cells—To determine whether VacA induces autophagic vacuoles, AZ-521 cells were incubated with 120 nM VacA. We microscopically observed that active VacA (A) is sufficient to trigger autophagic vacuoles such as autophagosomes containing LC3-II after a 4-h incubation, followed after by 12 h incubation by formation of autolysosomes as detected by LysoTracker (Fig. 4*A*). Cells incubated with heat-inactivated VacA (IA) showed low or undetectable levels of these autophagic vacuoles after 12 h incubation. Furthermore, confocal microscopy analysis showed that intracellular VacA partially co-localized with LC3-II and LRP1, consistent with the conclusion that LRP1 plays an important role in VacA-induced autophagosome formation. However, LRP1 knockdown with siRNA suppressed VacA co-localization with LC3-II, suggesting that LRP1 is essential for formation of autophagosomes in response to VacA (Fig. 4*B*).

Vacuoles Caused by VacA Are Characterized as Autophagosomes and Autophagolysosomes—Confocal microscope visualization of LC3-II, VacA, and LRP1 revealed that vacuoles caused by VacA are of at least two different types; one type consists of autophagic vacuoles such as autophagosomes and autophagolysosomes and the second type lacks LC3-II (Fig. 5*A*). These observations support previous findings that VacA-dependent autophagosomes and large vacuoles are distinct intracellular compartments and autophagy is independent of the formation of large vacuoles by VacA (29). Interestingly, some vacuoles observed with RPTPβ revealed small light vacuoles without LC3-II (Fig. 5*B*) and dense vacuoles with RPTPα were devoid of LC3-II (Fig. 5*C*). Although little is known about the physiological importance of the autophagy-dependent degradation of mitochondria (mitophagy) (39), several studies have suggested that PINK1/parkin-dependent mitophagy selectively degrades mitochondria (40), implying that mitophagy contributes to mitochondrial quality control. As shown in Fig. 5*D*, after 10 h incubation mitochondria were not observed in vacuoles with LC3-II. Furthermore, recent studies revealed that p62 binds to LC3 on the autophagosome membrane to target aggregates to autophagosomes for degradation (41). After 24 h incubation, VacA, not heat-inactivated VacA, induced formation of puncta, which were colocalized with LC3-II and p62 (Fig. 5*E*).

Among VacA-binding Proteins, LRP1, but Not RPTPs and FN, Mediates VacA-dependent Autophagy—To assess which VacA-binding proteins were responsible for VacA-induced autophagy, we examined the effect of silencing and knockout of the genes for RPTPβ, RPTPα, and fibronectin. Although LRP1 silencing blocked VacA-stimulated generation of LC3-II as shown in Fig. 3*b*, silencing these other genes did not show a similar effect, suggesting that only LRP1 may be critical for VacA-induced autophagy (Fig. 6).

LRP-1, but Not RPTPs, Mediates Cleavage of Caspase-7 and PARP Caused by VacA—Excessive autophagy can cause cell death (34, 42). Furthermore, VacA-induced cell death may occur through a programmed necrosis pathway in a caspase-independent process in AZ-521 cells (27). Therefore, we examined whether VacA-induced cell death resulted from autophagy via an LRP1-dependent pathway. Western blot analysis showed that LRP1 silencing blocked VacA-induced generation of LC3-II as well as cleavages of effector caspase-7 and PARP, suggesting that VacA binding to LRP-1 is responsible for not only autophagy but also for apoptosis in AZ-521 cells (Fig. 7).

Effects of Atg5 Silencing, Z-VAD-fmk and Necrostatin-1 on VacA-induced LC3-II Production and Cleavage of PARP—To further examine the link between autophagy and apoptosis, the effects of Atg5 silencing with siRNA, general caspase inhibitor (Z-VAD-fmk) and RIPK inhibitor (Necrostatin-1) on LC3-II generation, and PARP cleavage was evaluated. Silencing of the *Atg5* gene inhibited generation of LC3-II as well as PARP cleavages in response to VacA (Fig. 8), whereas both inhibitors, Z-VAD-fmk and Necrostatin-1, which interfere with apoptosis (43), did not inhibit VacA-induced autophagy, suggesting that VacA-induced autophagy precedes apoptosis in AZ-521 cells. Necrostatin-1, which inhibits necroptosis (44), did not interfere with VacA-induced generation of LC3-II and PARP cleavage.

Effect of Anion Channel Blockers, NPPB and DIDS, on VacA-induced LC3-II Production—To assess whether membrane channels formed by VacA may also be involved in autophagy (29), we tested the effects of pretreating AZ-521 or AGS cells with chloride channel blockers, NPPB and DIDS, which are known to block both VacA-mediated channel activity and cellular vacuolation (45). AZ-521 cells were pretreated for 30 min with 100 μM NPPB or 100 μM DIDS prior to incubation with VacA for 6 h. Both NPPB and DIDS inhibited VacA-induced LC3-II generation in AZ-521 cells (Fig. 9*a*), but not in AGS cells under these conditions (Fig. 9*b*).

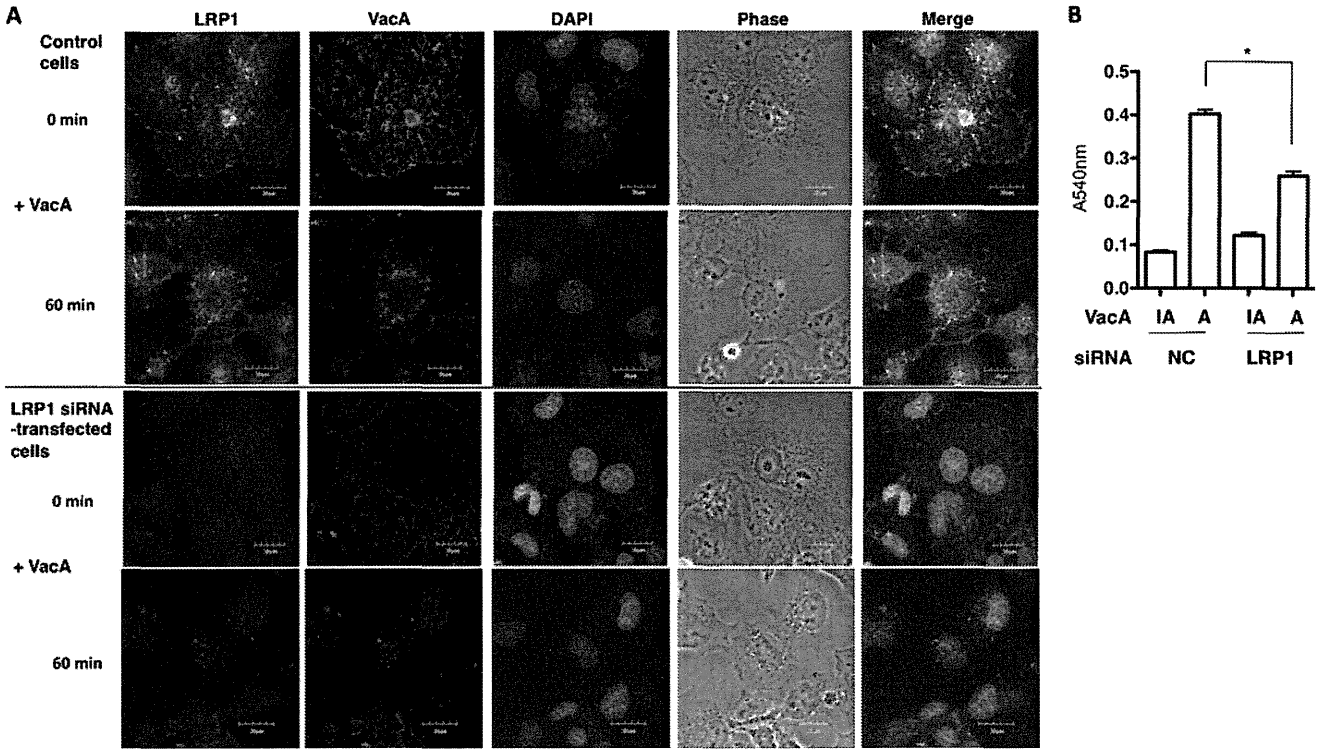


FIGURE 2. **LRP1-dependent VacA internalization and vacuolation in AZ-521 cells.** *A*, confocal microscopic analysis of VacA binding to AZ-521 cells via LRP1. Nontargeting (*NC*) or LRP1 siRNA-transfected AZ-521 cells were incubated with Alexa 555-labeled VacA (*red*) for 30 min at 4 °C or for 1 h at 37 °C, fixed with 4% paraformaldehyde, and reacted with anti-LRP1 antibodies (*green*) as described under "Experimental Procedures." The nuclei were stained with DAPI. A merged picture shows co-localization of VacA and LRP1 in AZ-521 cells. *Bars* represent 20 μm. Experiments were repeated two times with similar results. *B*, silencing of LRP1 gene inhibited VacA-induced vacuolation. The indicated siRNA-transfected AZ-521 cells were incubated with 120 nM heat-inactivated (*IA*) or wild-type VacA (*A*) for 18 h at 37 °C. Vacuolating activity was evaluated by neutral red uptake assay as described under "Experimental Procedures." Data are presented as mean ± S.D. and significance is (*) $p < 0.01$ ($n = 3$). Experiments were repeated three times with similar results.

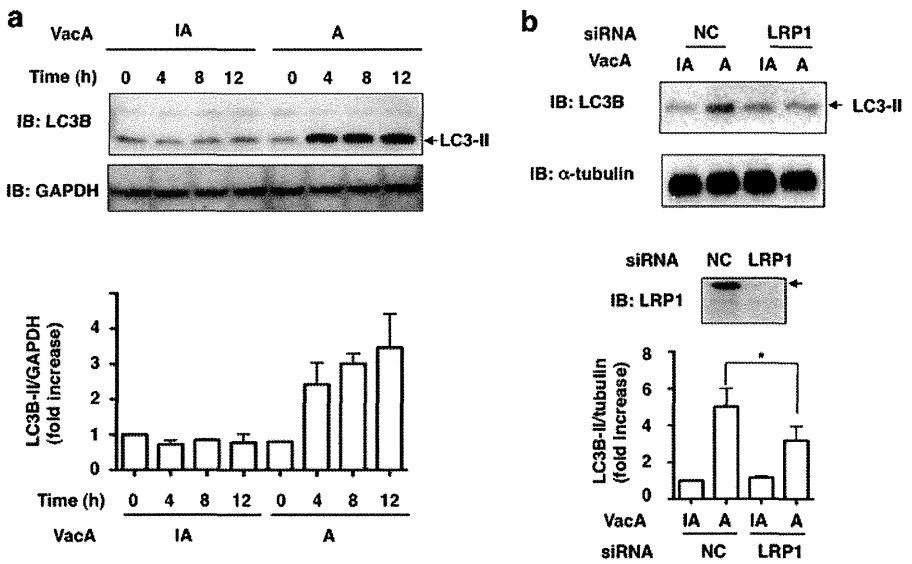


FIGURE 3. **VacA induced generation of LC3-II in an LRP1-dependent manner.** *a*, AZ-521 cells were incubated with 120 nM heat-inactivated (*IA*) or wild-type VacA (*A*) for the indicated time points and harvested for immunoblotting (*IB*) with the indicated antibodies. Quantification of VacA-induced LC3-II levels in AZ-521 cells was performed by densitometry (*bottom panel*). Data are presented as mean ± S.D. of values from two experiments. Experiments were repeated two times with similar results. *b*, the indicated siRNA-transfected AZ-521 cells were incubated with 120 nM heat-inactivated or wild-type VacA for 4–5 h at 37 °C and the cell lysates were subjected to immunoblotting with the indicated antibodies. α-Tubulin served as a loading control. Quantification of VacA-induced LC3-II levels in AZ-521 cells was performed by densitometry (*bottom panel*). Data are presented as mean ± S.D. and significance is (*) $p < 0.01$ ($n = 4$). Experiments were repeated four times with similar results.

DISCUSSION

VacA has two functional domains, an N-terminal 33.4-kDa domain (named p33, p34 or p37, comprising residues 1–311) and a C-terminal domain of 54.8 kDa (named p55 or p58, comprising residues 312–821) (10, 46, 47). Vacuolization of epithelial cells by VacA is strictly dependent on the formation of anion-selective membrane channels, which are targeted to late endosomes after internalization of the toxin (45, 48). The pore

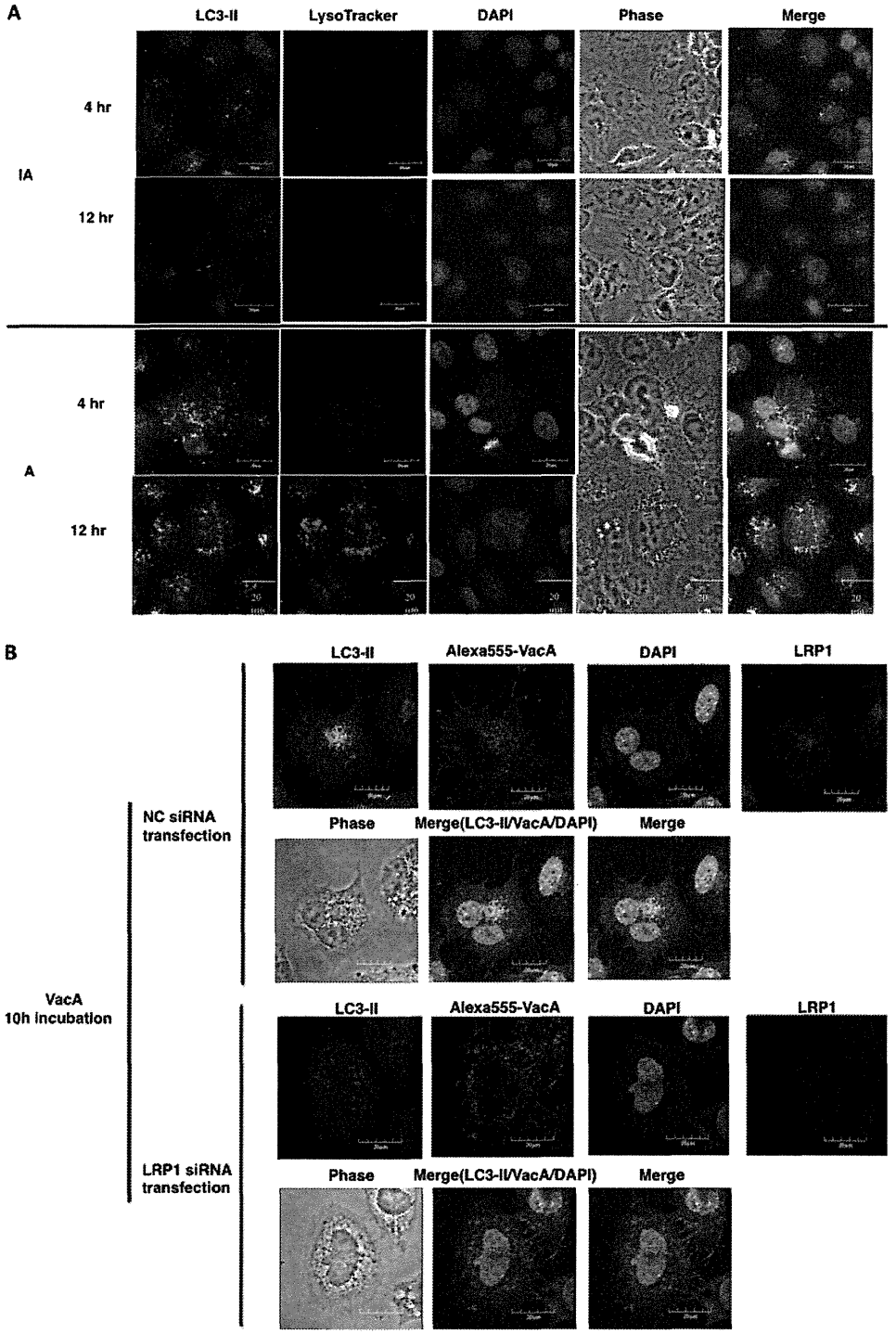
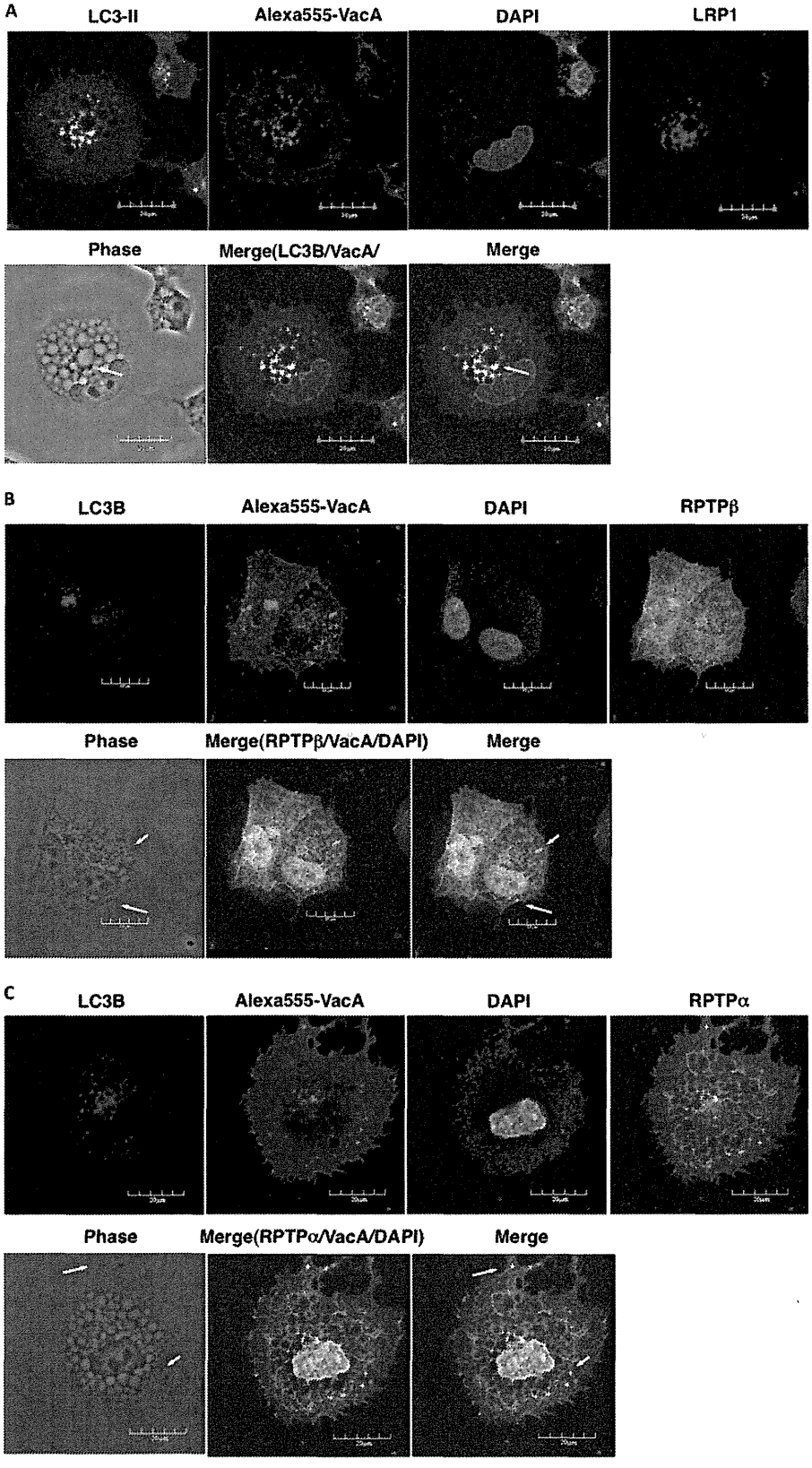


FIGURE 4. **VacA induced formation of autophagic vacuoles in AZ-521 cells via LRP1.** *A*, VacA-induced formation of autophagosomes and autolysosomes in AZ-521 cells. AZ-521 cells were incubated with 120 nM heat-inactivated (*IA*) or wild-type VacA (*A*) for the indicated time points and fixed for immunofluorescence staining with LC3B (*green*) antibodies as described under "Experimental Procedures." The acidic autophagolysosomes were stained by LysoTracker, as described under "Experimental Procedures." A merged picture shows co-localization in AZ-521 cells. The nuclei were stained with DAPI. *Bars* represent 20 μm. Experiments were repeated two times with similar results. *B*, induction of autophagy by VacA in an LRP1-dependent manner. The indicated siRNA-transfected AZ-521 cells were incubated with 120 nM Alexa 555-labeled VacA (*red*) for 10 h at 37 °C and fixed for immunofluorescence staining with anti-LC3B (*green*) or anti-LRP1 (*blue*) antibodies as described under "Experimental Procedures." A merged picture shows co-localization in AZ-521 cells. The nuclei were stained with DAPI. *Bars* represent 20 μm. Experiments were repeated two times with similar results.

and channel forming by the N-terminal p33 domain alone drives pleiotropic cellular activities of VacA; *i.e.* vacuolation, mitochondria damage, apoptosis (10, 47), autophagy (28, 29), and programmed necrosis (27), suggesting that VacA may be characterized as a pore-forming toxin (47). Another study has indicated that both p33 and p55 are required to form a functional channel in the inner mitochondria membrane and trigger apoptosis (49). In addition, it is now widely accepted that the C-terminal p55 domain of VacA plays an essential role in its binding to target cells (50, 51).

LRP1 Mediates VacA-induced Autophagy and Apoptosis

The present study defines a novel role for VacA signaling through LRP1 in AZ-521 cells, inducing autophagy and apoptosis (Fig. 7). LRP1 is a large endocytic receptor belonging to the LDL receptor family. This membrane protein consists of a 515-kDa heavy chain containing the extracellular ligand-binding domains and a noncovalently associated 85-kDa light chain, which consists of a transmembrane domain and a short cytoplasmic tail. LRP1 functions as a clearance receptor mediating



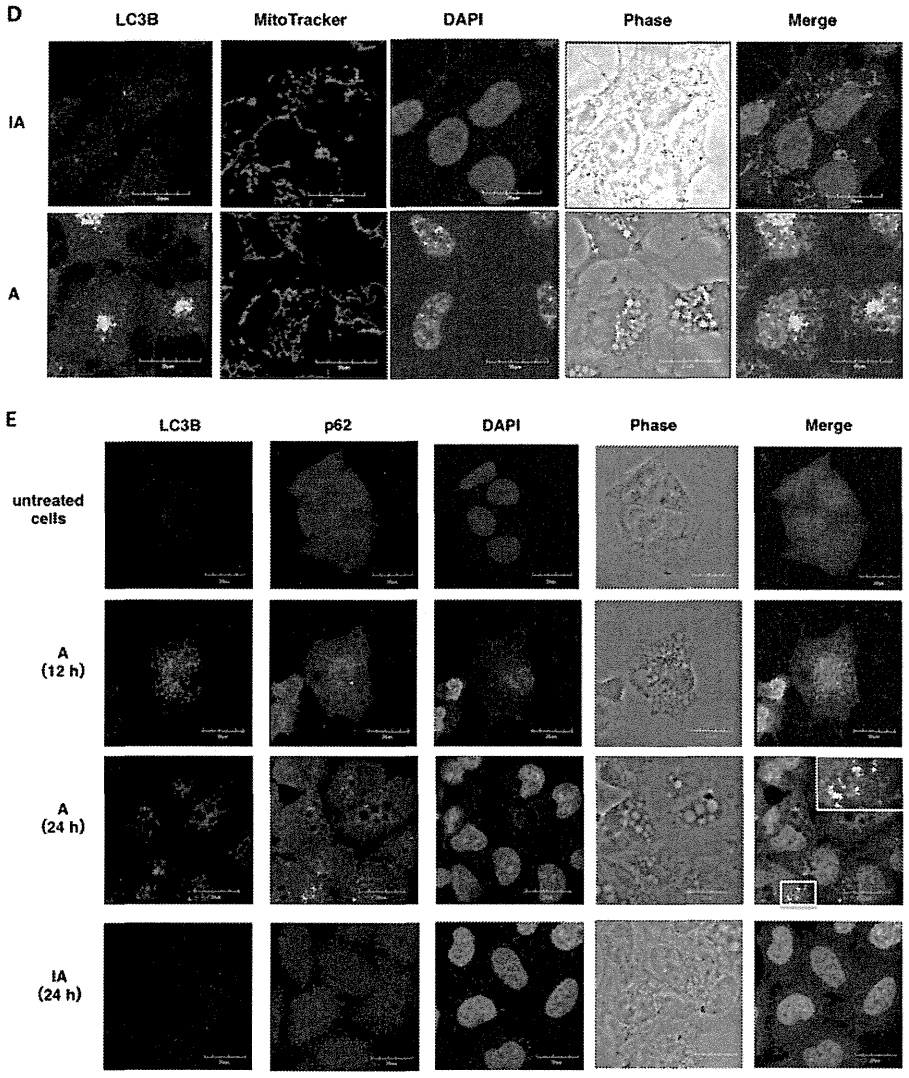


FIGURE 5—*continued*

the uptake and catabolism of various ligands from the pericellular environment, *e.g.* LRP1 binds to apolipoprotein E-rich lipoproteins, lipoprotein lipase, α_2-macroglobulin, lactoferrin, and tissue plasminogen activator; it functions in lipoprotein metabolism, degradation of proteases and proteinase/inhibitor complexes, activation of lysosomal enzymes and cellular entry of viruses, and bacterial toxin such as *Pseudomonas* exotoxin A (52). LRP1 has also been shown to function in the turnover of fibronectin (53).

This is the first study to provide evidence that LRP1 mediates autophagy. In AZ-521 cells, VacA triggered formation of autophagosomes, followed by autolysosome formation, consistent with the observations in AGS cells (29). Because LRP1 knockdown with siRNA resulted in inhibition of VacA-induced LC3-II generation and cleavage of both caspase 7 and PARP, induction by VacA of both autophagy and apoptosis occurred via, at least in part, association with LRP1. VacA also promoted formation of vacuoles containing RPTPβ and RPTPα, which

FIGURE 5. **Various vacuoles formed by VacA.** *A*, small autophagic vacuoles induced by VacA contain LC3-II, LRP1, and toxin: AZ-521 cells were incubated with 120 nM Alexa 555-labeled VacA (*red*) for 10 h at 37 °C and fixed for immunofluorescence staining with anti-LC3B (*green*), or anti-LRP1 (*blue*) antibodies or the nuclei were stained with DAPI as described under "Experimental Procedures." A merged picture shows co-localization in AZ-521 cells. *Solid arrows* show VacA, LC3B, and LRP1 colocalization to puncta. *Bars* represent 20 μm. Experiments were repeated two times with similar results. *B*, VacA-induced light vacuoles contain toxin and RPTPβ, and are different from autophagic vacuoles: AZ-521 cells were treated with 120 nM Alexa 555-labeled VacA (*red*) as similar to above. Cells were fixed and stained for anti-LC3B (*blue*), anti-RPTPβ (*green*), and with DAPI. A merged picture shows co-localization in AZ-521 cells. *Solid arrows* show VacA and RPTPβ colocalization to puncta. *Bars* represent 20 μm. Experiments were repeated two times with similar results. *C*, VacA-induced dense vacuoles contain toxin and RPTPα, and are different from autophagic vacuoles: AZ-521 cells were treated with 120 nM Alexa 555-labeled VacA (*red*) as similar to above. Cells were fixed and stained for anti-LC3B (*blue*), anti-RPTPα (*green*), and with DAPI. A merged picture shows co-localization in AZ-521 cells. *Solid arrows* show VacA and RPTPα colocalization to puncta. *Bars* represent 20 μm. Experiments were repeated two times with similar results. *D*, VacA-induced autophagic vacuoles do not contain functional mitochondria: AZ-521 cells were treated with 120 nM heat-inactivated (*IA*) or native VacA (*A*) for 10 h at 37 °C and 100 nM MitoTracker (*red*) was added to cells before fixation as described under "Experimental Procedures." Cells were stained for anti-LC3B (*green*), anti-p62 (*green*), and with DAPI. *Bars* represent 20 μm. Experiments were repeated two times with similar results. *E*, VacA induced p62 generation in a time-dependent manner. AZ-521 cells were treated with 120 nM heat-inactivated or native VacA for the indicated time points at 37 °C. Cells were fixed and stained for anti-LC3B (*red*) and with DAPI. Merged and higher magnification images of the outlined areas are shown. *Bars* represent 20 μm. Experiments were repeated two times with similar results.