and 1:4), Sadato et al (4) evaluated the histopathology at several time intervals after embolization. Their results showed no recanalization of the vessels in which embolization was achieved, although it was not disclosed how far distally the embolic material traveled. Other studies reported the relationship between the number of arteries in which embolization was achieved and the ischemic changes in the intestinal tract wall according to different ratios of NBCA/Lip (5,6). However, no study had quantified the relationship between the NBCA/Lip ratio and the degree of the embolic effect or how far distally the embolic material reached.

We conducted the present study to examine quantitatively the relationship of property in vitro (viscosity, diffusing capacity, and polymerization time) and embolization effect in vivo of various ratios of NBCA/Lip. In the in vitro study, which used a more modern and precise method with a high-speed camera, we confirmed more objectively that the polymerization time increased with the Lip volume, which was indicated in previous experimental studies using traditional methods (7–9). The difference in the polymerization time according to the differential ratio might have resulted because it occurred only where the NBCA/Lip came into contact with blood but not in the remaining part where it was separate from the blood. Lip could have inhibited the contact between NBCA and blood and polymerization. We presume that the actual polymerization time during transcatheter arterial embolization might be shorter than the results in our in vitro study because of more likely contact between NBCA/Lip and blood.

In the in vivo experiment with histopathologic measurement of number and axis of arterioles, embolization occurred more distally at a 1:3 ratio than at a 1:1 ratio. However, no significant difference was found between 1:3 and 1:9 ratios in vivo, where polymerization time and viscosity greatly changed in vitro. The exact reason for this finding is unclear, but there might have been some interaction of the peripheral arterial embolization effect with viscosity within an extremely narrowed lumen; increased viscosity owing to a high ratio of Lip may disproportionately reduce blood flow within small arterioles or capillaries, resulting in a relatively limited degree of peripheral embolization. This aspect was not investigated in our in vitro study. The kinetics of NBCA/Lip may be modified by not only polymerization time and viscosity but also other factors, including blood vessel diameter, blood velocity, and blood pressure and their combination, which remain to be evaluated.

Similar to histopathologic measurement, the cortical vascular bed that underwent embolization (not including the medullary part of the kidney) measured by CT volumetry was significantly greater with NBCA/Lip ratios of 1:3 and 1:9 than a ratio of 1:1, although quantity of NBCA/Lip injected was not significantly different among the three groups. This finding may indicate that the medullary (not cortical) vascular bed that underwent embolization was greater at the mixture ratio of 1:1 than the ratios of 1:3 and 1:9.

It was presumed from the in vitro results on viscosity and diffusing capacity that NBCA/Lip likely flows more peripherally in blood vessels in vivo when the NBCA density is decreased. In our in vivo study, although no significant difference was found between 1:3 and 1:9 ratios, both the number of arteries in which embolization was achieved on pathologic examination (**Fig E3** [available online at *www.jvir.org*]) and the vascular bed in which embolization was achieved on CT volumetry (**Fig E1** [available online at *www.jvir.org*]) were larger with the ratio of 1:9 than with 1:3. These results may contradict the opinion that NBCA/Lip does not easily reach peripheral arteries when the Lip density is increased. Based on the in vitro results of polymerization time and in vivo results, we suggest using the ratio of less dense NBCA (closer to 1:9) when there remains some distance between the microcatheter tip and the target to avoid premature or too proximal embolization. Information acquired in this study on the relationship between the NBCA/Lip ratio and intravascular polymerization factors would help decide the ratio in individual cases in terms of safe and effective embolization.

The limitations of this study include our examinations only of NBCA/Lip ratios of 1:1–1:9, as these are frequently clinically used. We were able to determine the relationships of the viscosity, the polymerization time, and the embolism effect in this range. However, we did not confirm whether similar relationships are maintained with a different range of ratios.

In conclusion, we quantitatively examined the relationship of properties of NBCA/Lip in vitro and embolization effects in vivo of various ratios of NBCA/Lip and compared the data. The results of this study are useful for safe and effective embolization.

ACKNOWLEDGMENT

The authors acknowledge Yasuhiro Nakamura, Fumiyoshi Fujishima, Kazuhiro Sato, and Tomoyoshi Kimura.

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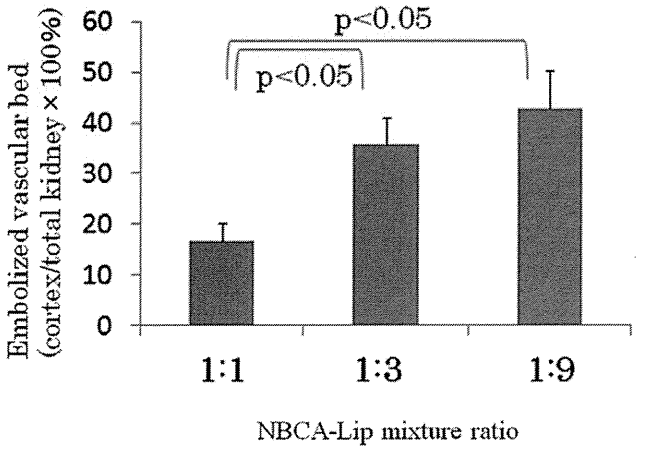

Figure E1. The embolized cortical vascular bed was increased as the Lip volume increased. The vascular bed was significantly greater with NBCA/Lip ratios of 1:3 and 1:9 than with an NBCA/Lip ratio of 1:1, whereas no significant difference was observed between NBCA/Lip ratios of 1:3 and 1:9.

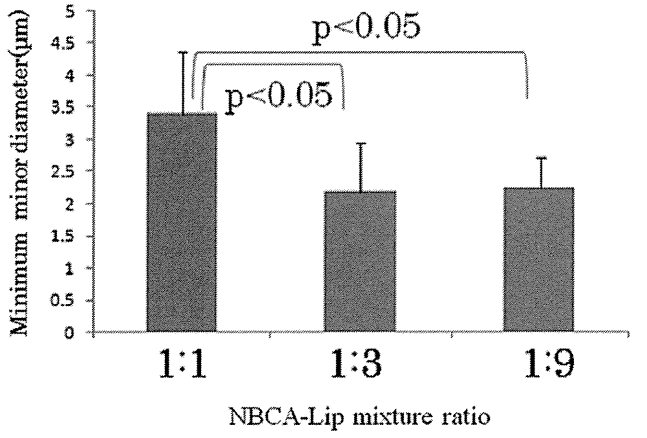

Figure E2. The minimum minor diameter of the arteriole containing embolic material in the lumen was measured in each kidney. With ratios of 1:3 and 1:9, the embolic material was found in significantly narrower arterioles than at a 1:1 ratio.

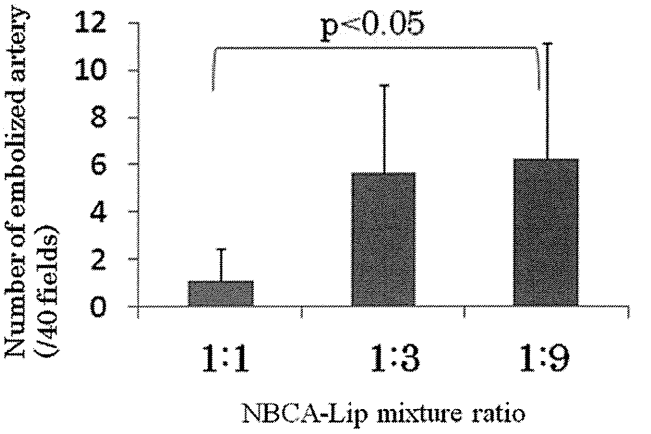

Figure E3. The number of arterioles with a minor diameter $\leq 40\ \mu m$ containing embolic material in the lumen was counted. At a low NBCA density, narrow arterioles were embolized, and the peripheral distribution of the embolic material tended to be broad. More arterioles were embolized at a 1:9 ratio than significantly at 1:1.

Okamoto et al. World Journal of Surgical Oncology 2013, 11:51
http://www.wjso.com/content/11/1/51

WORLD JOURNAL OF SURGICAL ONCOLOGY

RESEARCH Open Access

Significance of CD133 expression in esophageal squamous cell carcinoma

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Abstract

Background: CD133 was recently reported to be a cancer stem cell marker and a prognostic marker for several tumors. However, few studies have investigated CD133 expression in esophageal squamous cell carcinoma (ESCC). Therefore, we examined whether CD133 could serve as a prognostic marker of ESCC and investigated the correlation between CD133 expression and the clinicopathological findings of ESCC patients and several markers.

Methods: We studied 86 ESCC patients who underwent curative surgery without neoadjuvant treatment at Tohoku University Hospital (Sendai, Japan) between January 2000 and December 2005. We analyzed tissue specimens by immunohistochemical staining for CD133, p53, p16, p27, murine double minute 2 (MDM2), Ki-67, and epidermal growth factor receptor (EGFR).

Results: Pathological tumor depth and tumor stage were significantly more advanced among CD133-negative patients than among CD133-positive patients. A log-rank test showed that CD133 immunoreactivity was significantly correlated with the overall survival of the patients ($P = 0.049$). However, multivariate analysis showed that it was not significantly correlated ($P = 0.078$). Moreover, CD133 was significantly positively correlated with p27 immunoreactivity ($P = 0.0013$) and tended to be positively correlated with p16 immunoreactivity ($P = 0.057$). In addition, p16 immunoreactivity was correlated with smoking history ($P = 0.018$), pathological lymph node status ($P = 0.033$), and lymphatic invasion ($P = 0.018$).

Conclusions: This study indicated that CD133 immunoreactivity is a good predictor of prognosis in ESCC patients. In addition, CD133 may play a role in the regulation of tumor cell cycle through p27 and p16 in ESCC. At present, it thus remains controversial whether CD133 expression is a valid prognostic marker for ESCC. To elucidate this relationship, further investigations are required.

Keywords: AC133, Esophagus, Prominin-1, p16, p27, Stem cell marker

Background

Prognosis or clinical outcome of esophageal squamous cell carcinoma (ESCC) has markedly improved over the last several decades, owing to advancements in medical treatment. However, in Japan, 11,867 people succumbed to this disease in 2010, and esophageal cancer was the seventh most common cause of cancer mortality in men (3.4% of the total cancer deaths in Japan) [1]. Various prognostic markers have recently been evaluated, including the stem cell marker CD133 (Prominin-1), which was reported to be a cancer stem cell marker for cancers of the brain [2], colon [3,4], prostate [5], liver [6,7], lung [8], kidney [9], ovaries [10], and skin [11,12]. It was also reported to be a marker of poor prognosis for cancers of the brain and spinal cord [13], colon [14], rectum [15], pancreas [16], breast [17], and stomach [18]. In addition, a potent cytotoxic drug, monomethyl auristatin F, which acts as an anti-CD133 antibody-drug conjugate for hepatocellular and gastric cancer cells, may be utilized to treat CD133-positive tumors [19]. To date, there have

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been limited studies of CD133 in esophageal cancer, and thus, the significance of CD133 in this form of cancer remains unclear. Therefore, we examined whether CD133 could serve as a prognostic marker of ESCC. In addition, we explored the correlation between CD133 expression and the clinicopathological findings of ESCC patients and the correlation between CD133 expression and the immunolocalization of several markers, such as p53, p16, p27, murine double minute 2 (MDM2), Ki-67, and epidermal growth factor receptor (EGFR), which are known as prognostic markers or tumor proliferation factors in ESCC [20-27].

Methods

Patients and tissue samples

A total of 86 consecutive ESCC patients, who underwent curative surgery without neoadjuvant treatment at Tohoku University Hospital (Sendai, Japan) between January 2000 and December 2005, were selected. All patients underwent thoracoscopic esophagectomy with two- or three-field node dissection, except for four patients who underwent pharyngo-laryngo-esophagectomy with one-field node dissection, six patients who underwent transhiatal esophagectomy with one-field node dissection, and eight patients who underwent esophagectomy by right thoracotomy.

The resected specimens and lymph nodes were fixed in 10% formalin, and representative sections were embedded in paraffin wax. The sections were histologically examined according to the Union for International Cancer Control (UICC) TNM (tumor, node, metastasis) classification (7th edition) system [28]. Patient survival time was determined from the date of surgery until death, recurrence, or the last follow-up examination. This study was approved by the ethical committee of the Tohoku University Hospital (Accession number 2011–596).

Immunohistochemical staining and evaluation

Serial sections (4 μm thick), including the deepest area of the tumors, were deparaffinized in xylene, rehydrated in graded alcohol, and immersed in 3.0% hydrogen peroxide in methanol for 10 min at room temperature (RT) to inhibit endogenous peroxidase activity. For antigen retrieval, the slides for p53 were heated by microwave irradiation at 95°C for 15 min in 0.01 M citrate buffer (pH 6.0). The slides for p16, p27, MDM2, and Ki-67 were heated by autoclave at 121°C for 5 min in 0.01 M citrate buffer (pH 6.0). The slides for CD133 were autoclaved at 121°C for 5 min in Histofine antigen retrieval solution (pH 9.0, Nichirei Biosciences Inc., Tokyo, Japan). The slides for EGFR were incubated in 0.05% protease in Tris–HCl buffer (pH 7.6) at 37°C for 10 min. After washing three times for 5 min each in phosphate-buffered saline (PBS), the slides were incubated in 1% normal rabbit serum for 30 min at RT to reduce nonspecific antibody binding and were subsequently incubated at 4°C overnight with mouse monoclonal antibody against p53 (DO-7, Leica Microsystems, Bannockburn, IL, USA, diluted 1/100), p16 (G175-1239, BD Biosciences, Franklin Lakes. NJ, USA, diluted 1/100), p27 (SX53G8, Dako, Glostrup, Denmark, diluted 1/800), MDM2 (SMP14, Santa Cruz Biotechnology Inc., CA, USA, diluted 1/1000), Ki-67 (MIB-1, Dako, diluted 1/300), EGFR (31G7, Nichirei Biosciences Inc., dilution unknown, product code 413701), CD133 (AC133, Miltenyi Biotec, Auburn, CA, USA, diluted 1/10). The following day, the sections were washed three times for 5 min each in PBS, incubated with biotinylated anti-mouse immunoglobulin (Nichirei Biosciences Inc.), washed three times for 5 min each in PBS, and incubated with peroxidase-labeled streptavidin (Nichirei Biosciences Inc.) for 30 min at RT. The immunohistochemical signal was visualized with 3,3′-diaminobenzidine, and the slides were counterstained with Mayer's hematoxylin, dehydrated in graded alcohol, and cleared in xylene. For CD133, omission of the primary antibody and substitution by nonspecific immunoglobulin (Mouse IgG1, Dako) at the same concentration were used as negative and isotype controls, respectively.

The sections were examined by two independent observers (HO and FF) who were blinded to patients' clinical information. The proportion of positive nuclei in more than 1,000 tumor cells of more than three fields under a ×400 magnification microscope (Leica DM LB2) at the deepest area of each tumor was calculated for p53, p27, MDM2, and Ki-67. The proportion of nuclei and cytoplasm of tumor cells positive for p16 was evaluated. The proportion of membranes of tumor cells positive for EGFR was evaluated. The cut-off values for abnormal expression were as follows: p53, ≥10% [21]; p16, ≤5% [26]; p27, ≥10% [25]; MDM2, ≥20% [29]; and Ki-67, ≥30% [27]. An immunoreactive score (IRS) was used for the scoring of EGFR. The IRS is obtained by multiplying the intensity score (0, no staining; 1, faint staining; 2, moderate staining; 3, strong staining) by the extent score (0, none; 1, <10%; 2, 10 to 50%; 3, >50 to 80%; 4, >80%) and ranges from 1 to 12. An IRS of ≥6 was defined as positive for EGFR expression [20]. When evaluating CD133, the tumors were defined as positive and negative when >1% and ≤1% of the membranes and cytoplasm of all tumor cells were immunostained, respectively [13,30].

Statistical analyses

Statistical analyses were performed using JMP Pro Version 9.0.2 (SAS Institute Inc., Cary, NC, USA). The correlation of factors was evaluated by the chi-square test, Fisher's exact test, or Wilcoxon test, as appropriate. Survival curves were determined by the Kaplan-Meier

method, and differences in survival between groups were compared by the log-rank test. The Cox proportional hazard model was used for multivariate analysis. A P value of <0.05 was considered statistically significant.

Results

Correlation between CD133 and clinicopathological findings of patients

Table 1 summarizes the clinicopathological findings of the patients examined. The median follow-up time was 69.0 months (range, 1 to 149 months). The patients included 73 men and 13 women with a median age of 64 years (range, 37 to 81 years). The number of patients in each pathological stage was as follows: 20, pStageI; 28, pStageII; 33, pStageIII; and 5, pStageIV. There were five patients with M1 lymph nodes. Of the 86 patients, 38 (44.2%) were immunohistochemically positive for CD133 (Figure 1). pT and pStage were significantly more advanced among CD133-negative patients compared with CD133-positive patients (Table 1).

Correlation between CD133 and other markers

Table 2 summarizes the correlation between expression of CD133 and expression of other molecular markers examined. CD133 and p27 expression were positively correlated (P = 0.0013), and CD133 and p16 expression tended to be positively correlated (P = 0.057) but did not reach statistical significance. No significant correlations

Table 1 Correlation between clinicopathological findings and CD133 status

		Total (%)	CD133 expression		P
			Positive	Negative	
			n = 38 (44.2%)	n = 48 (55.8%)	
Age (years)	Mean ± SD	63.9 ± 9.4	64.8 ± 10.9	63.2 ± 8.0	0.45
	(Range)	(37–81)	(37–9)	(44–81)	
Sex	Male	73 (84.9)	32 (84.2)	41 (85.4)	0.88
	Female	13 (15.1)	6 (15.8)	7 (14.9)	
Smoking	Absent	13 (15.1)	8 (21.1)	5 (10.4)	0.17
	Present	73 (84.9)	30 (79.0)	43 (89.6)	
Location	Cervix	5 (5.8)	3 (7.9)	2 (4.2)	0.59
	Upper	5 (5.8)	1 (2.6)	4 (8.3)	
	Middle	35 (40.7)	17 (44.7)	18 (37.5)	
	Lower	41 (47.7)	17 (44.7)	24 (50.0)	
Histological type	Well	17 (19.8)	9 (23.7)	8 (16.7)	0.68
	Moderate	56 (65.1)	23 (60.5)	33 (68.8)	
	Poor	13 (15.1)	6 (15.8)	7 (14.6)	
pT	pT1	28 (32.6)	19 (50.0)	9 (18.8)	0.017
	pT2	9 (10.5)	3 (7.9)	6 (12.5)	
	pT3	46 (53.5)	15 (39.5)	31 (64.6)	
	pT4	3 (3.5)	1 (2.6)	2 (4.2)	
pN	pN0	36 (41.9)	19 (50.0)	17 (35.4)	0.17
	pN1-3	50 (58.1)	19 (50.0)	31 (64.6)	
pM	pM0	81 (94.2)	35 (92.1)	46 (95.8)	0.65
	pM1 (LYM)	5 (5.8)	3 (7.9)	2 (4.2)	
pStage	pStageI	20 (23.3)	14 (36.8)	6 (12.5)	0.035
	pStageII	28 (32.6)	9 (23.7)	19 (39.6)	
	pStageIII	33 (38.4)	12 (31.6)	21 (43.8)	
	pStageIV	5 (5.8)	3 (7.9)	2 (4.2)	
Lymphatic invasion	Negative	34 (39.5)	13 (34.2)	21 (43.8)	0.37
	Positive	52 (60.5)	25 (65.8)	27 (56.3)	
Venous invasion	Negative	31 (36.1)	11 (29.0)	20 (41.7)	0.22
	Positive	55 (64.0)	27 (71.1)	28 (58.3)	

SD, standard deviation.

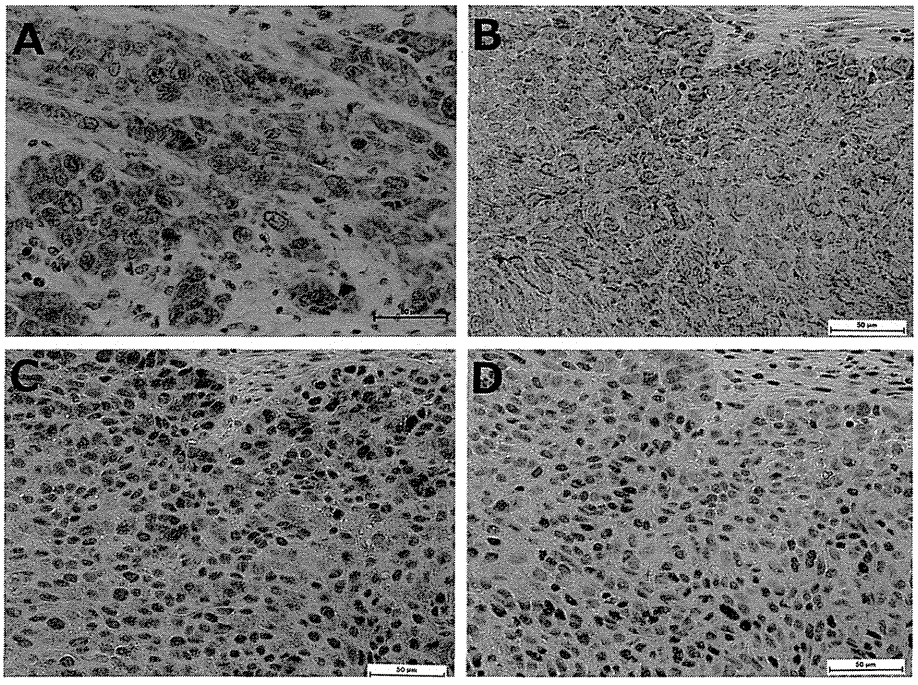

Figure 1 Immunohistochemical staining of esophageal squamous cell carcinoma. Tumor cells positive for CD133 **(A,B)**, p16 **(C)**, and p27 **(D)** expression (×400 magnification). In addition, **B**, **C**, and **D** were at the same site of the same tumor.

were detected between expression of CD133 and expression of any other marker.

Correlations for other molecular markers

In terms of correlations between the other molecular markers and clinicopathological findings, p16 expression was correlated with smoking history (P = 0.018), pathological lymph node status (P = 0.033), and lymphatic invasion (P = 0.018) (Additional file 1). With regard to correlations among other molecular markers, p53 expression was positively correlated positively with Ki-67 expression (P = 0.0030) (Additional file 2).

Survival analysis

The 3- and 5-year survival rates of all patients examined were 65.0% and 61.5%, respectively. Results of univariate analysis of postoperative overall survival (OS) and disease-free survival (DFS) are summarized in Table 3.

Table 2 Correlation between expression of CD133 and expression of other molecular markers

		Total (%)	CD133 expression		*P*
			Positive	Negative	
			n = 38 (44.2%)	*n* = 48 (55.8%)	
p53	Negative	29 (33.7)	13 (34.2)	16 (33.3)	0.93
	Positive	57 (66.3)	25 (65.8)	32 (66.7)	
p16	Negative	69 (80.2)	27 (71.1)	42 (87.5)	0.057
	Positive	17 (19.8)	11 (29.0)	6 (12.5)	
p27	Negative	37 (43.0)	9 (23.7)	28 (58.3)	0.0013
	Positive	49 (57.0)	29 (76.3)	20 (41.7)	
MDM2	Negative	66 (76.7)	31 (81.6)	35 (72.9)	0.35
	Positive	20 (23.3)	7 (18.4)	13 (27.1)	
Ki-67	<30	43 (50.0)	17 (44.7)	26 (54.2)	0.39
	≥30	43 (50.0)	21 (55.3)	22 (45.8)	
EGFR	Negative	46 (53.5)	23 (60.5)	23 (47.9)	0.24
	Positive	40 (46.5)	15 (39.5)	25 (52.1)	

Table 3 Univariate survival analysis of clinicopathological findings and expression of molecular markers

	Variables	Number	Overall survival		Disease-free survival	
			5-year overall survival rate (%)	*P*	5-year disease-free survival rate (%)	*P*
Age (years)	≤60	29	72.4	0.11	62.1	0.21
	>60	57	55.9		52.5	
Sex	Male	73	58.7	0.13	51.9	0.080
	Female	13	76.9		76.9	
Location	Cervix/upper	10	50.0	0.12	40.0	0.067
	Middle/lower	76	63.0		57.8	
Histological type	Well or moderate	73	61.5	0.64	57.4	0.36
	Poor	13	61.5		46.2	
pT	pT1/pT2	37	78.1	0.0012	70.0	0.0033
	pT3/pT4	49	49.0		44.9	
pN	pN0	36	80.6	0.0051	75.0	0.0078
	pN1-3	50	47.7		41.7	
pStage	pStageI/II	48	79.0	0.0002	72.8	0.0002
	pStageIII/VI	38	39.5		34.2	
Lymphatic invasion	Negative	34	70.2	0.086	58.4	0.29
	Positive	52	55.8		53.9	
Venous invasion	Negative	31	64.5	0.47	54.8	0.63
	Positive	55	59.8		56.2	
p53	Negative	29	62.1	0.30	55.2	0.55
	Positive	57	61.3		56.1	
p16	Negative	69	58.0	0.19	53.6	0.14
	Positive	17	76.0		63.7	
p27	Negative	37	56.8	0.30	54.0	0.49
	Positive	49	65.0		56.9	
MDM2	Negative	66	63.6	0.31	59.1	0.61
	Positive	20	53.3		59.2	
Ki-67	<30	43	69.8	0.24	62.8	0.27
	≥30	43	53.2		48.6	
EGFR	Negative	46	69.6	0.22	63.0	0.16
	Positive	40	52.0		47.1	
CD133	Negative	48	54.2	0.049	47.9	0.059
	Positive	38	70.8		65.5	

Overall survival was significantly correlated with pT, pN, pStage, and CD133 status, and was significantly longer in CD133-positive patients than in CD133-negative patients (P = 0.049) (Figure 2). No significant correlation between OS and the other markers was observed (Figure 3). Multivariate analysis demonstrated that pStage was a significant prognostic factor for OS and that pStage and tumor location were significant prognostic factors for DFS. Correlation between CD133 expression and patient survival did not reach statistical significance by multivariate analysis (Table 4).

Discussion

CD133 was originally identified as a transmembrane glycoprotein in normal hematopoietic stem and progenitor cells [31] that participated in proliferation, self-renewal, and multilineage differentiation [32]. Furthermore, CD133 was recently used to identify putative cancer stem cells of several tumors [33]. According to several studies, CD133 was associated with tumor differentiation in several organs [16,34-36]. For example, Jiang *et al.* [36] reported that CD133 expression was increased in diffuse-type gastric cancers compared with intestinal-type cancers and was increased more so in poorly differentiated than in moderately

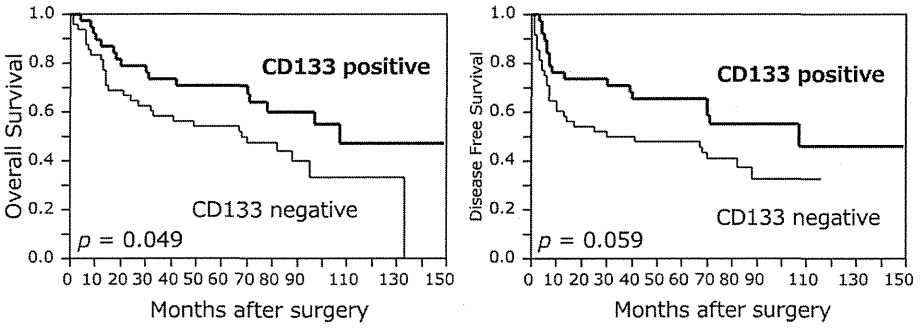

Figure 2 Kaplan-Meier curves of patients with esophageal squamous cell carcinoma according to CD133 expression. Overall survival was significantly longer in CD133-positive patients than in CD133-negative patients ($P = 0.049$). There was no significant correlation between disease-free survival and CD133 status ($P = 0.059$).

or well differentiated gastric cancers. In addition, Feng *et al.* [35] reported that CD133 was negatively correlated with the cellular differentiation status of colon cancer cells. Finally, Fan *et al.* [34] reported that CD133 expression was correlated with well differentiated or moderately differentiated cholangiocarcinomas and that subcellular CD133 localization was correlated with the tumor differentiation status. In terms of ESCC, Hang *et al.* reported that CD133 expression was increased in well differentiated and moderately differentiated ESCCs compared with poorly differentiated ESCCs [30]. However, in our study, no correlation was detected between CD133 expression and tumor differentiation of carcinoma cells. We think that this was because there is a difference among pathologists or facilities regarding the histological evaluation of tumor differentiation status, and in addition, our study was small. On the other hand, CD133 expression correlated with p27 expression ($P = 0.0013$) and tended to correlate with the status of p16 immunoreactivity ($P = 0.057$). The relationship between CD133 and cell cycle regulators has remained unclear in esophageal cancer. There may be a correlation between CD133 and cell cycle pathways associated with the INK4 family or the

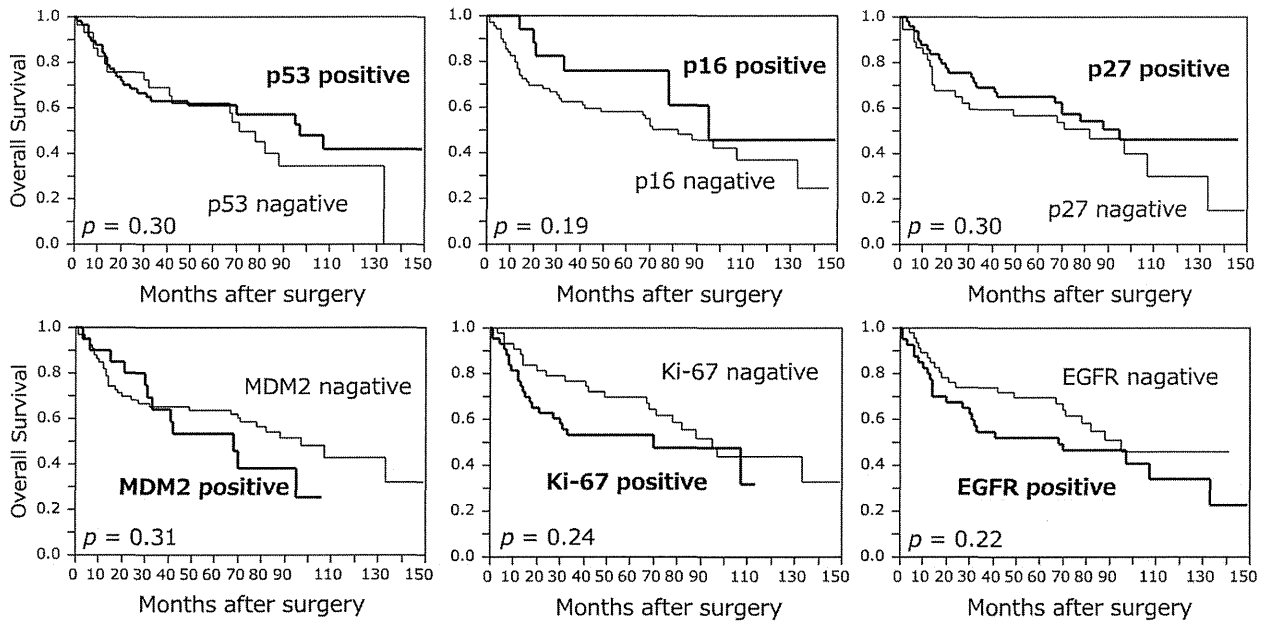

Figure 3 Kaplan-Meier curves of patients with esophageal squamous cell carcinoma according to expression of the other markers. No significant correlation between overall survival and the other markers was observed.

Table 4 Multivariate survival analysis of clinicopathological findings and expression of molecular markers

	Variables	Hazard ratio	95% confidence interval	*P*
Overall survival	pStageIII/IV	2.64	1.42-5.03	0.0020
	Lymphatic invasion positive	1.48	0.79-2.89	0.23
	CD133 negative	1.74	0.94-3.35	0.078
Disease-free survival	Male	2.09	0.84-6.99	0.12
	Cervix/upper	2.51	1.07-5.21	0.035
	pStageIII/IV	2.93	1.62-5.42	0.0004
	CD133 negative	1.73	0.96-3.24	0.071

CIP/KIP family of cyclin-dependent kinase inhibitors [37], but this possibility requires further investigation.

To the best of our knowledge, there are few reports that have investigated the effect of CD133 expression on survival of ESCC patients. Nakajima *et al.* [38] reported that CD133 expression in resected ESCC specimens following neoadjuvant chemoradiotherapy tended to be correlated with poor prognosis, but multivariate analysis did not produce a significant correlation. In contrast, CD133 expression was significantly correlated with poor response to neoadjuvant chemoradiotherapy. Hang *et al.* [30] reported that CD133 expression in resected ESCC specimens without preoperative treatment was not significantly correlated with prognosis. Our study revealed that OS was significantly longer in CD133-positive patients than in CD133-negative patients, as determined by log-rank test. One reason for this was that the tumors were significantly more advanced (according to their pStage classification) in CD133-negative patients than in CD133-positive patients. Although correlation between CD133 expression and patient survival did not reach statistical significance, as determined by multivariate analysis, CD133 immunoreactivity may have the potential to be a good predictor of prognosis in ESCC patients. With regard to other tumors, CD133 expression in non-small cell lung cancers [39,40], hepatocellular carcinomas [18], and pancreatic cancers [41] was not correlated with patient survival. Moreover, CD133-negative expression in cholangiocarcinomas was correlated with poor prognosis [34], which is similar to that revealed in our study. Further studies are needed to clarify this issue.

Conclusions

In conclusion, this study demonstrated that CD133 immunoreactivity may have the potential to be a good predictor of prognosis in ESCC patients, and that CD133 may play a role in the regulation of tumor cell cycle through p27 and p16 in ESCC. At present, whether CD133 expression is a valid prognostic marker for ESCC remains controversial. To elucidate this relationship, further investigations are required, including verification of an evaluation method for CD133 immunoreactivity in ESCC.

Consent

Written informed consent concerning the procedure of this study was obtained from all patients prior to study enrollment.

Additional files

Additional file 1: Correlation between clinicopathological findings and p16 status. p16 expression was correlated with smoking history ($P = 0.018$), advanced pN ($P = 0.033$), and lymphatic invasion ($P = 0.018$).

Additional file 2: Correlation between p53 status and Ki-67 status, MDM2 status. p53 expression was positively correlated with Ki-67 expression ($P = 0.0030$).

Abbreviations

DFS: Disease-free survival; EGFR: Epidermal growth factor receptor; ESCC: Esophageal squamous cell carcinoma; IRS: Immunoreactive score; MDM2: Murine double minute 2; OS: Overall survival; PBS: Phosphate-buffered saline; RT: Room temperature; TNM: Tumor, node, metastasis; UICC: Union for International Cancer Control.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HO is the main author of this article. HO and FF conceived this study. FF and YN supervised the manuscript writing. MZ, YO, GM, TK, TN, YT, and JT contributed to the collection of clinical information and data analysis. HO and YT performed the experiments. HO and FF performed the pathological examination and immunohistochemical evaluation. MW, AS, NO, and HS reviewed the manuscript and revised it thoroughly. All authors have read and approved the final manuscript.

Acknowledgements

We thank the staff of the Department of Pathology, Tohoku University Hospital for their excellent technical assistance and Enago (www.enago.jp) for an English language review.

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Received: 23 August 2012 Accepted: 12 February 2013
Published: 1 March 2013

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doi:10.1186/1477-7819-11-51
Cite this article as: Okamoto *et al.*: Significance of CD133 expression in esophageal squamous cell carcinoma. *World Journal of Surgical Oncology* 2013 11:51.

Dig Dis Sci (2013) 58:2266–2274
DOI 10.1007/s10620-013-2660-7

ORIGINAL ARTICLE

Reactive Increase in Gastric Mucus Secretion Is an Adaptive Defense Mechanism Against Low-Dose Aspirin-Induced Gastropathy

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Received: 9 January 2013 / Accepted: 20 March 2013 / Published online: 7 May 2013

Abstract
Background Gastric mucus is considered to play an essential role in gastric mucosal defense mechanisms, especially when irritants are present in the stomach.
Aim To investigate the relationship between low-dose aspirin-induced gastropathy and gastric secretory function, especially gastric mucus secretion, in healthy volunteers.
Methods Thirty male, asymptomatic, *Helicobacter pylori pylori*-negative healthy volunteers were asked to take 100 mg of enteric-coated aspirin (Bayaspirin) once a day for 10 days. Endoscopic examination was performed before and 3 and 10 days after drug administration. The extent of endoscopically assessed gastric mucosal injury was semi-quantitatively evaluated according to the modified Lanza score. The pentagastrin-stimulated gastric juice was collected for 10 min during the endoscopic examination and subjected to analysis for gastric acid (mEq/10 min) or mucus (mg hexose/10 min) output.
Results Overall, the 10-day aspirin treatment significantly increased gastric mucus secretion from 0.8 (interquartile range 1.7) to 1.6 (1.6) mg hexose/10 min ($P < 0.05$), with a concomitant and significant decrease in the gastric acid/mucus ratio from 4.3 (5.2) to 2.9 (4.7) ($P < 0.01$). Subsequent analysis of two subgroups of volunteers categorized according to their endoscopic status ("severe gastropathy" vs. "modest gastropathy") revealed that changes in gastric secretory parameters occurred exclusively in those subjects without severe gastric injury; there was no alteration in these parameters in subjects with severe gastric injury.
Conclusions The results of this study suggest that the reactive increase in gastric mucus secretion is an adaptive defense mechanism against low-dose aspirin-induced gastropathy. In some individuals, such a response may be insufficient to prevent the development of severe mucosal injury and even ulcers and their complications.

Keywords Low-dose aspirin · Gastropathy · Gastric adaptation · Gastric mucus secretion

Abbreviations

COX	Cyclooxygenase
EGT	Endoscopic gastrin test
GSRS	Gastro-intestinal symptom rating scale
LDA	Low-dose aspirin
MLS	Modified Lanza score
NSAID	Non-steroidal anti-inflammatory drug

Introduction

Aspirin has long been used as an effective antipyretic analgesic in the treatment of a wide spectrum of conditions and diseases. It is also now widely administered at relatively low doses (100 mg/day in most Japanese patients) as an antithrombotic drug for the prevention of cerebrovascular and cardiovascular disease. Despite the definite

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benefits of these antithrombotic effects, aspirin, even at a low dose, is known to cause upper gastro-intestinal (GI) complications, such as hemorrhagic gastritis and gastroduodenal ulcers [1, 2]. Our current understanding of the gastric mucosa is that its integrity is dependent on an equilibrium between aggressive factors and protective mechanisms [3–5]. Consequently, a good understanding of how these factors contribute to aspirin-induced epithelial disruption is important for establishing a preventive strategy for upper GI complications.

The gastric mucus serves as the first line of mucosal defense against noxious luminal acid by creating an unstirred layer on the mucosal surface that both supports the maintenance of a near-neutral pH at the surface and acts as a physical barrier [3–5]. The turnover of surface mucus biosynthesis can ultimately be reflected by an increase or decrease in the amount of soluble mucus secreted in the gastric lumen [6, 7]. In clinical study of patients taking long-term low-dose aspirin (LDA) for cardiovascular and/or cerebrovascular disease, we recently found that the level of gastric mucus secretion was significantly higher in chronic LDA-takers than in non-aspirin controls [8]. In addition, the higher level of mucus secretion was more prominent in LDA-takers without severe gastric injury than in those with severe gastric injury [8]. Based on the results of this observational study, we speculated that the higher level of mucus secretion in LDA-takers might be a reactive response to repetitive oral administration of LDA and that the reactive mucus secretion might play a pivotal role in gastric mucosal protection against luminal irritants. However, to prove that gastric mucus secretion is actually changed by the administration of lDA, we needed to conduct a subsequent study to measure gastric mucus secretion both prior to and after the administration of LDA in an appropriate subject group, such as healthy volunteers.

It is well-known that the gastric mucosa can become more tolerant or adaptive in response to the prolonged administration of noxious agents, such as aspirin [9]. In a number of studies in humans, a high dose of aspirin (1–2.5 g per day) was shown to consistently induce maximal gastric injury within 3 days of treatment initiation, but thereafter the lesions tended to resolve despite continued administration of the drug [9–11]. However, the precise mechanism of this adaptation to aspirin under depleted prostaglandin biosynthesis due to the inhibition of the cyclo-oxgenase (COX) [12, 13] enzyme remains to be clarified. In addition, although the aspirin-induced gastric mucosal injury and subsequent adaptive response showed a dose–response relationship [14], few time-course studies on LDA-induced gastric mucosal injury have been reported [15, 16].

In this study involving healthy volunteers, we performed a time-course study to monitor gastric mucosal injury and gastric secretory parameters during a 10-day administration of 100 mg aspirin. Our aim was to determine the relationship between the gastric mucosal adaptive response and gastric secretory parameters.

Methods

Thirty male asymptomatic and healthy volunteers (mean age 26.7 years, range 19–42 years), all of whom were nonsmokers were enrolled in this study. All subjects had *Helicobacter pylori pylori*-negative status determined by the ^{13}C-urea breath test. None of the subjects had a history of peptic ulcer or *H. pylori* eradication, and none was taking any drugs before entry into the study or during the study period. All subjects were asked to take 100 mg of enteric-coated aspirin (Bayaspirin, Bayer, Tokyo) once in the morning each day for 10 days. Each participant underwent three endoscopic examinations: one before (day 0) and two (on day 3 and day 10) after the initiation of drug administration. Each endoscopic examination was performed after an overnight fast, and the drug was administered 2 h before the examination. In each endoscopic examination, gastric secretory function was evaluated with the endoscopic gastrin test (EGT) technique described below, and the extent of endoscopically assessed gastric mucosal injury was semi-quantitatively evaluated. The study was approved by the Tohoku University School of Medicine Ethics Committee (2009-356), and each subject gave written informed consent.

Gastric Aspiration with the EGT Technique

The details of EGT have been reported previously [17]. Briefly, the subjects were injected intramuscularly with pentagastrin at a dose of 6 μg/kg (pentagastrin; Sigma, St. Louis, MO) about 15 min before the endoscopic examination. Following insertion of the endoscope into the stomach, the gastric fluid which had pooled in the stomach was aspirated and discarded. The gastric juice newly secreted between 20 and 30 min after the pentagastrin injection and which pooled in the upper part of the participant's stomach, with the individual lying in a left lateral decubitus position, was aspirated and collected under direct visualization during the routine endoscopic examination. During the examination, the subjects were asked not to swallow their saliva. After the collection of gastric juice, the endoscope was removed. The volume of gastric juice collected in the 10-min period was recorded, and the collected gastric juice was then divided into two aliquots, with one aliquot subjected to analysis for gastric acid secretion and the other to analysis for gastric mucus secretion. The laboratory investigators were blinded to the subjects' medical information.

Assessment of Gastric Acid Secretion

The H^+ concentration of the gastric juice was determined by titration. Acid output in the 10-min period was calculated by multiplying the volume by the H^+ concentration, and the EGT value was expressed as milliequivalents per 10 min. We have previously shown that the EGT values correlate very well with the peak acid output determined by conventional methods (correlation efficient 0.92) with high reproducibility (coefficient of variation 5.6 %) [17].

Extraction and Isolation of Mucin in Gastric Juice

The collected gastric juice was centrifuged at 1,500 *g* for 30 min at room temperature to remove contaminating debris. The mucin, a major component of gastric mucus, in the gastric juice samples was extracted and isolated using a previously described method which enables mucin to be successfully isolated and condensed from the gastric juice without contamination by non-mucin glycoproteins, such as serum-type glycoproteins. Absolute ethanol (6 mL) was added to 2 mL of the supernatant obtained from the gastric juice to a final concentration of 75 % ethanol (v/v). The resultant suspension was maintained at 4° overnight to complete the precipitation, after which the precipitate was collected by centrifugation (8,000 *g* for 30 min at 4 °C). The pellet was dissolved in distilled water (2 mL), and its hexose content was measured by the phenol–sulfuric acid method. The mucin content of the gastric juice was expressed as the amount of hexose in the solution obtained by the precipitation method (μg/mL). The total mucus output was determined by multiplying the mucin concentration by the volume of gastric juice collected in the 10-min period [18].

Endoscopic Evaluation of Gastric Mucosal Injury

The grade of gastric mucosal injury was assessed according to the modified Lanza score (MLS) [19, 20]. In our analysis, the scoring system was based on the number of erosions and/or ulcers despite the presence or absence of hemorrhaging because petechial hemorrhages are generally of little clinical significance [21]. Gastric mucosal injury was graded into six groups, ranging from 0 to 5, with grade 0 = no erosion, grade 1 = one to two lesions of erosion localized in one area of the stomach, grade 2 = three to five lesions of erosion localized in one area of the stomach, grade 3 = six to nine lesions of erosion localized in one area of the stomach or no more than ten lesions in two areas of the stomach, grade 4 = erosions in three areas of the stomach or no fewer than ten lesions in the whole stomach, and grade 5 = a gastric ulcer defined as a mucosal defect >5 mm in diameter.

During endoscopy, more than 40 endoscopic pictures covering the whole area of the stomach were saved in the database. These were subsequently used by two endoscopists (KI, TI), who were blinded to other information on the subjects, to independently grade the MLS. In cases of disagreement, a consensus was reached through joint review of the endoscopic pictures.

Evaluation of Gastrointestinal Symptoms

Gastrointestinal symptoms were assessed using the GI symptom rating scale (GSRS) [22]. The GSRS contains 15 items rated on the 7-point Likert scale, where score 1 represents "no discomfort" and score 7 represents "very severe discomfort." The items have been divided into five major GI symptoms: abdominal pain, reflux, indigestion, diarrhea, and constipation. The GSRS was used before each endoscopic examination and the scores were averaged for each major GI symptom.

Statistics

Based on our recent report [8], the number of subjects required to detect an absolute difference in gastric mucus secretion at a two-sided alpha level of 0.05 with 80 % power was calculated to be $n = 26$. In addition to various gastric secretory parameters derived directly from the determination of the gastric aspirates, we calculated the ratio of total acid output relative to total mucus output (acid/mucus ratio), which reflects the importance of the equilibrium between aggressive and protective factors in the pathogenesis of gastric mucosal injury [8]. Because the distribution of some raw data was considerably skewed, continuous data as well as non-parametric data were expressed as the median and inter-quantile range (IQR), and the statistical significance of differences was determined by the Wilcoxon rank sum test. A P value of <0.05 was considered to be statistically significant.

Results

Of the 30 subjects who were initially enrolled in this study, one individual was subsequently excluded from the analysis due to a moderate level of endoscopically assessed gastric mucosal injury at entry. The remaining 29 subjects completed the study protocol without problematic side effects. In all gastric aspirations with the EGT technique, a sufficient amount of gastric fluid was collected for the analysis of both gastric acid and mucus secretion.

Endoscopic Gastric Injury

The administration of 100 mg aspirin to healthy volunteers induced a variety of endoscopic gastric mucosal reactions. While the aspirin did not cause any discernible mucosal damage in some of the volunteers, it did cause severe mucosal damage, even gastric ulceration, in others during the experimental period. Overall, the median MLS showed a modest but significant increase by 3 days after first administration of the drug, from 0.0 (IQR 1.0) at day 0 to 1.0 (IQR 1.0) at day 3 ($P < 0.01$); thereafter, there was no further increase in the MLS between days 3 and 10 [1.0 (IQR 1.0) vs. 1.0 (IQR 1.3); not significant) (Fig. 1a; Table 1).

Since a wide range of gastric mucosal injuries were observed following the administration of LDA, all subjects were subsequently divided into one of two subgroups according to their endoscopic status on day 10—i.e., one group comprising subjects (23/29, 79 %) showing only modest endoscopic evidence of mucosal damage (MLS ≤ 2 on day 10) and the other group comprising subjects (6) with severe gastric mucosal injury (MLS ≥ 4 at day 10) (there was no subject presenting a MLS of 3 on day 10). Further analysis revealed that there were substantial differences in the time-course of LDA-induced gastropathy between the two subgroups. Subjects with modest gastric injury showed an initial significant increase in MLS from 0.0 (IQR 1.0) on day 0 to 1.0 (IQR 1.0) on day 3; thereafter, however, the score tended to decrease, reaching 1.0 (IQR 1.5) on day 10 ($P = 0.11$); this MLS on day 10 was no longer significantly different from that on day 0 (Fig. 1b; Table 2). Subjects with severe gastric injury also showed an initial, modest increase in MLS from 0.0 (IQR 1.0) on day 0 to 1.5 (IQR 2.0) on day 3 ($P < 0.05$); however, in contrast to the subjects with modest gastric injury, these subjects showed a further prominent increase in MLS to 4.0 (IQR 0) on day 10 ($P < 0.05$) (Fig. 1c; Table 2).

Gastric Secretory Parameters

The analysis of all the participants showed that the gastric secretory volume and acid secretion level were unchanged after 10 days of LDA administration, but that the mucus secretion level steadily increased during this time, although it showed a relatively skewed distribution. Overall, the median LDA-induced increase in gastric mucus output rose from 0.8 (IQR 1.7) mg hexose/10 min on day 0 to 1.1 (IQR 1.2) mg hexose/10 min on day 3 and to 1.6 (IQR 1.6) mg hexose/10 min on day 10, with the difference reaching statistical significance on day 10 compared with day 0 ($P = 0.02$) (Fig. 2a; Table 1). Together with the increase in mucus secretion, LDA administration resulted in a consistent decrease in the gastric acid/mucus ratio, from 4.3 (IQR 5.2) on day 0, to 3.6 (IQR 5.6) on day 3, and finally to 2.9 (IQR 4.7) on day 10, with the difference reaching statistical significance on day 10 compared with day 0 ($P = 0.008$) (Fig. 2b; Table 1).

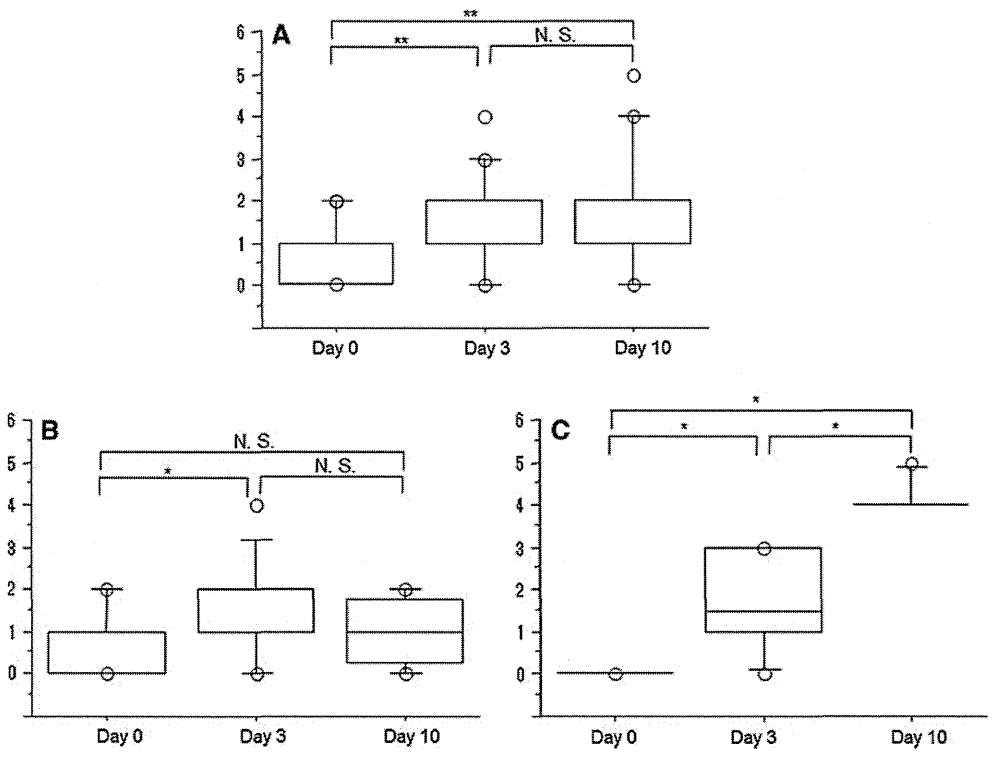

Fig. 1 Time-course of modified Lanza score during the 10-day administration of 100 mg aspirin. Data are shown for the entire subject cohort (**a**), for those with modest gastric injury on day 10 (**b**), and for those with severe gastric injury on day 10 (**c**). $*P < 0.05$, $**P < 0.01$, *N.S.* not significant

Table 1 Change in various parameters during the 10-day administration of low-dose aspirin among the entire study cohort

Parameters	Day 0	Day 3	Day 10	*P* value
Epigastric pain score	1.0 (0.3)	1.0 (0.4)	1.0 (0.7)	Day 0 vs. day 3: $P = 0.82$
				Day 3 vs. day 10: $P = 0.65$
				Day 0 vs. day 10: $P = 0.31$
Modified Lanza score	0.0 (1.0)	1.0 (1.0)	1.0 (1.3)	Day 0 vs. Day 3: $P = 0.002$
				Day 3 vs. day 10: $P = 0.31$
				Day 0 vs. day 10: $P = 0.002$
Secretion volume (mL)	36.0 (8.9)	33.0 (13.0)	33.0 (7.0)	Day 0 vs. day 3: $P = 0.60$
				Day 3 vs. day 10: $P = 0.39$
				Day 0 vs. day 10: $P = 0.010$
Total acid output (mEq/10 min)	4.3 (1.5)	4.3 (1.2)	4.4 (1.1)	Day 0 vs. day 3: $P = 0.68$
				Day 3 vs. day 10: $P = 0.97$
				Day 0 vs. day 10: $P = 0.49$
Total mucus output (mg hexose/10 min)	0.8 (1.7)	1.1 (1.2)	1.6 (1.6)	Day 0 vs. day 3: $P = 0.80$
				Day 3 vs. day 10: $P = 0.12$
				Day 0 vs. day 10: $P = 0.02$
Gastric acid/mucus ratio	4.3 (5.2)	3.6 (5.6)	2.9 (4.7)	Day 0 vs. day 3: $P = 0.38$
				Day 3 vs. day 10: $P = 0.14$
				Day 0 vs. day 10: $P = 0.009$

All data are expressed as the median, with the inter-quartile range (IQR) given in parenthesis

When the same analyses of gastric secretory parameters were repeated in the two subgroups described above, the changes in the parameters showed a pattern that differed depending on the extent of injury. Subjects with modest gastric injury showed a steadily increasing trend in gastric mucus output during the observation period, with the median value increasing from 0.8 (IQR 1.6) mg hexose/10 min on day 0, to 1.1 (IQR 1.1) on day 3, and finally to 1.4 (IQR 1.7) on day 10, with the difference reaching statistical significance on day 10 compared with day 0 ($P = 0.02$). In contrast, mucus output in subjects with severe gastric injury remained at a similar level during the observation period, with median values of 1.9 (IQR 1.1) mg hexose/10 min on day 0, 1.8 (IQR 0.9) mg hexose/10 min on day 3, and 1.9 (IQR 0.8) mg hexose/10 min on day 10 (Table 2). There was no significant alteration in the gastric acid secretion level during the observation period regardless of the extent of gastric mucosal injury. Consequently, the gastric acid/mucus ratio steadily decreased in subjects with modest gastric injury during the 10-day administration of LDA, with median ratios of 5.2 (IQR 7.6) on day 0, 3.8 (IQR 5.6) on day 3, and 2.9 (IQR 4.9) on day 10; the difference was statistically significant between day 0 and day 10 ($P = 0.009$). In contrast, there was no trend of alteration in the acid/mucus ratio in subjects with severe gastric injury during the observation period; the median ratio was 3.1 (IQR 0.5) on day 0, 2.1 (IQR 1.9) on day 3, and 3.0 (IQR 1.8) on day 10 (Table 2). Due to the small number of subjects with severe gastric injury ($n = 6$) and the relatively scattered data, we failed to find any significant difference in the relative change in mucus output or acid/mucus ratio between subjects with and without severe gastric injury [1.1 (IQR 1.1) vs. 1.3 (IQR 0.9), respectively, for mucus output on day 10 relative to the pre-treatment value of 0.9 (IQR 0.5) vs. 0.7 (IQR 0.5) for acid mucus ratio; $P > 0.2$ for each].

In addition, there was no significant difference in the pre-treatment (day 0) values of the gastric secretory parameters between those with and those without severe gastric mucosal injury, although gastric acid secretion and mucus output tended to be higher in those with severe gastric injury compared to those without the injury (Table 2).

Symptom Score

There was no discernible change in the epigastric pain score or other abdominal symptom scores during the observation period irrespective of the extent of endoscopic gastric mucosal injury (Tables 1, 2).

Discussion

In this study, we sought to determine the relationship between LDA-induced gastropathy and gastric secretory function, especially gastric mucus secretion, by monitoring these parameters using an endoscopic aspiration technique

Table 2 Changes in various parameters during the 10-day administration of low-dose aspirin among subgroups based on the manifestation of endoscopic gastric mucosal injury

Parameters	Subjects with modest gastropathy ($n = 23$)				Subjects with severe gastropathy ($n = 6$)			
	Day 0	Day 3	Day 10	P value	Day 0	Day 3	Day 10	P value
Epigastric pain score	1.0 (0.6)	1.0 (0.6)	1.0 (0.7)	Day 0 vs. day 3: $P = 0.93$ Day 3 vs. day 10: $P = 0.75$ Day 0 vs. day 10: $P = 0.50$	1.0 (0.3)	1.0 (0)	1.0 (0.7)	Day 0 vs. day 3: $P = 0.99$ Day 3 vs. day 10: $P = 0.65$ Day 0 vs. day 10: $P = 0.29$
Modified Lanza score	0.0 (1.0)	1.0 (1.0)	1.0 (1.5)	Day 0 vs. day 3: $P = 0.02$ Day 3 vs. day 10: $P = 0.11$ Day 0 vs. day 10: $P = 0.08$	0.0 (1.0)	1.5 (2.0)	4.0 (0)	Day 0 vs. day 3: $P = 0.04$ Day 3 vs. day 10: $P = 0.03$ Day 0 vs. day 10: $P = 0.02$
Secretion volume (mL)	36.0 (9.4)	31.5 (12.1)	31.5 (7.1)	Day 0 vs. day 3: $P = 0.83$ Day 3 vs. day 10: $P = 0.11$ Day 0 vs. day 10: $P = 0.08$	40.0 (7.5)	33.5 (15.5)	39.0 (9.0)	Day 0 vs. day 3: $P = 0.23$ Day 3 vs. day 10: $P = 0.34$ Day 0 vs. day 10: $P = 0.60$
Total acid output (mEq/10 min)	4.1 (1.4)	4.3 (1.1)	4.2 (1.0)	Day 0 vs. day 3: $P = 0.96$ Day 3 vs. day 10: $P = 0.55$ Day 0 vs. day 10: $P = 0.93$	5.3 (1.9)	4.5 (2.9)	5.2 (1.3)	Day 0 vs. day 3: $P = 0.46$ Day 3 vs. day 10: $P = 0.46$ Day 0 vs. day 10: $P = 0.17$
Total mucus output (mg hexose/10 min)	0.8 (1.6)	1.1 (1.1)	1.4 (1.7)	Day 0 vs. day 3: $P = 0.92$ Day 3 vs. day 10: $P = 0.12$ Day 0 vs. day 10: $P = 0.02$	1.9 (1.1)	1.8 (0.9)	1.9 (0.8)	Day 0 vs. day 3: $P = 0.46$ Day 3 vs. day 10: $P = 0.92$ Day 0 vs. day 10: $P = 0.60$
Gastric acid/mucus ratio	5.2 (7.6)	3.8 (5.6)	2.9 (4.9)	Day 0 vs. day 3: $P = 0.48$ Day 3 vs. day 10: $P = 0.09$ Day 0 vs. day 10: $P = 0.009$	3.1 (0.5)	2.1 (1.9)	3.0 (1.8)	Day 0 vs. day 3: $P = 0.34$ Day 3 vs. day 10: $P = 0.92$ Day 0 vs. day 10: $P = 0.46$

All data are expressed as the median, with the IQR given in parenthesis

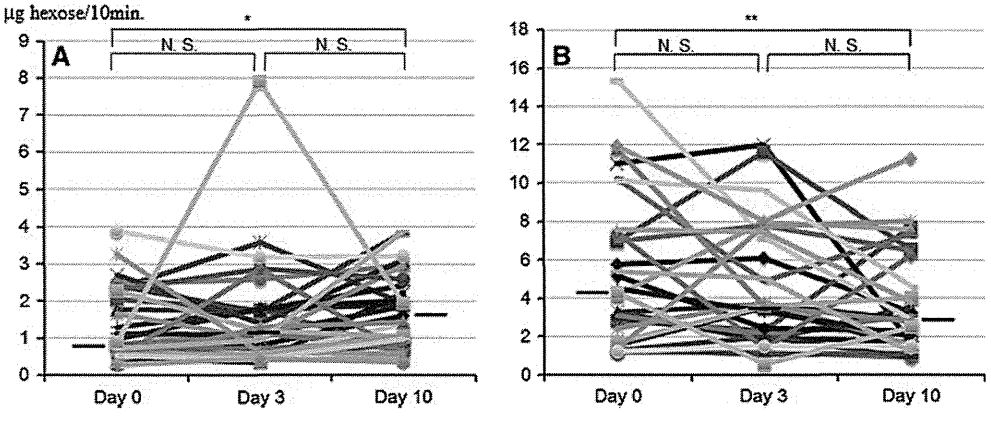

Fig. 2 Time-course of gastric mucus output (**a**) and gastric acid/mucus ratio (**b**) during the 10-day administration of 100 mg aspirin among the entire study cohort. * $P < 0.05$, ** $P < 0.01$, *N.S.* not significant. *Dark horizontal bars* Median values

in a cohort of healthy volunteers receiving daily doses of LDA for 10 days. In the study cohort overall, LDA administration significantly increased gastric mucus secretion with a concomitant significant decrease in the gastric acid/mucus ratio. Interestingly, such changes in gastric secretory parameters were observed exclusively in those subjects without severe gastric injury, while there was no alteration in these parameters in the subjects with severe gastric injury. Thus, our results suggest that a reactive increase in gastric mucus secretion may be physiologically significant as an adaptive defense mechanism against LDA-induced gastropathy.

Earlier studies with high doses of aspirin demonstrated that gastric injury peaked at 3 days of treatment at an intensive level, followed by a tendency for the lesions to resolve despite continued administration of the drug [9–11, 14]. However, in more recent observational studies with LDA of 7-day duration, gastric injury was found to be relatively modest and to reach a plateau at 3 days of treatment [15, 16]. Our results are consistent with those of these more recent studies [15, 16] and demonstrate that, overall, LDA induced a modest but significant increase in gastric mucosal injury on day 3 in our healthy volunteers, with the level of injury remaining relatively constant up to day 10. Nonetheless, we could recognize two different subgroups of subjects based on the time-course of the gastric injury during LDA administration. Although both groups showed an initial, significant increase in gastric injury on day 3, the patterns were different thereafter. One group, comprising the majority (79 %) of subjects, showed a tendency for resolution of the injury between day 3 and day 10, while the other group, comprising a minority (21 %) of the subjects, showed a further exacerbation of the injury during the same period. This finding that not all subjects exhibited sufficient adaptive resolution of the injury is consistent with the results of an earlier study [13]. Hence, this study reveals that whereas the stomach of the majority of our subjects adapted to repeated administration of LDA, this typical gastric response failed in a small number of our subjects.

Early animal model studies produced controversial results with respect to the effect of aspirin administration on gastric mucus secretion, with some studies showing that the thickness of the gastric gel mucus significantly increased following exposure to aspirin [23, 24], while others showed that aspirin inhibited the biosynthesis of mucus [25], gastric mucosal mucus content [26, 27], and/or secretion of the gastric mucus [28]. Yet another study demonstrated recovery of the gastric mucosal mucin several hours after a significant decrease in secretion immediately following instillation of aspirin [29]. Different methodological approaches could be partly responsible for these inconsistent results. Nonetheless, these animal model studies are difficult to extrapolate to the human situation of repeated doses of the drug, because other than the potential inter-specific differences, most of these animal studies dealt with the results from observations of only several hours after a single instillation of aspirin [23–29]. Meanwhile, there have been very few human studies that have investigated the relationship between repeated aspirin intake and gastric mucus secretion [14]. To our knowledge, this is the first study that has examined the effect of repeated LDA administration and gastric mucus secretion in human subjects. Our results reveal a significant, overall increase in gastric mucus secretion in our healthy volunteers following 10 days of LDA administration.

The substantial increase in gastric mucus secretion by day 10 of LDA administration in our healthy volunteers seems to contradict a previous study describing that a 1-week administration of naproxen decreased total mucin output in *H. pylori*-negative healthy volunteers [30]. Differences in the doses of the employed drugs might explain these different results, as would the different type of drugs [aspirin vs. other nonsteroidal anti-inflammatory drugs (NSAIDs)]. Although both aspirin and other NSAIDs similarly deplete gastric prostaglandin synthesis by the inhibition of the COX enzyme [12, 13, 21], these aspirin and other NSAIDs are known to differently affect two types of COX enzyme isoforms. While ordinary NSAIDs inhibit both COX-1 and COX-2, aspirin preferentially inhibits COX-1. There is a possibility that, unlike other NSAIDs, aspirin can uniquely affect gastric mucus secretion.

Our analysis of the two subgroups of study subjects based on the extent of gastric mucosal injury revealed a significant increase in gastric mucus secretion only in those who showed an adaptive response that resolved the mucosal injury in the later phase of the study; in contrast, mucus secretion was unchanged in those subjects who failed to show the adaptive response and experienced further gastric injury. This finding seems to be consistent with those of previous studies in rats showing that the initial aspirin-induced reduction in gastric mucosal mucin was followed by subsequent recovery of the mucin [29] and that the mucin content was significantly correlated with the extent of aspirin-induced gastric damage [31]. In contrast, baseline values (day 0) of gastric mucus secretion tended to be paradoxically lower in subjects without severe gastric injury compared to those with injury, although not significantly so. Thus, a reactive increase in gastric mucus secretion following exposure to LDA—rather than a steady-state secretory level—is more likely to be important in terms of acting as a protective covering that inhibits further deterioration of focally injured areas. However, this process may fail in some individuals who go on to develop severe mucosal injury, even ulcers, and related

complications. Additionally, previous studies have shown that NSAIDs and aspirin increase gastric epithelial cell exfoliation [32, 33]. Since gastric epithelial cells contain large amounts of mucus, one may consider that an increased amount of mucus in the collected gastric juice in our study subjects was due to the exfoliation by the aspirin administration rather than any increase in mucus secretion. However, such scenario is refuted by the findings that the amount of mucus tended to increase more in subjects with the least endoscopically assessed injury.

Of relevance to our findings in healthy volunteers receiving LDA over the short term (10 days), we recently demonstrated in the clinical setting of long-term LDA-takers (>2 years of intake in the majority of cases) that the gastric mucus secretion level was significantly higher in aspirin-takers than in the non-aspirin controls and that the increased level of mucus secretion was more prominent in aspirin-takers without severe gastric injury than in those with severe gastric injury [8]. Taken together, it is likely that the changes in gastric secretory function in response to apparent gastric mucosal injury observed in the present short-term study could persist during prolonged administration, suggesting that our findings have clinical implications. To date, the intricate mechanisms by which gastric mucosal adaptation to aspirin and other NSAIDs occurs are not fully understood, although the process entails an increase in mucosal blood flow [11, 13, 34] and enhancement in mucosal defense by increased cellular proliferation [14, 35, 36]. Otherwise, the results of our study suggest that an increase in mucus secretion in response to damaging agents could have an important role in gastric adaptation. Since our study cohort consisted of a relatively homogenous population of healthy volunteers (relatively young, male, *H. pylori*-negative, non-smokers), further studies regarding genetic polymorphisms responsible for gastric mucus secretion independently of prostaglandin synthesis are warranted to determine the susceptibility of LDA-takers to drug-induced gastropathy. Among prostaglandin-independent pathways, growth factors, such as transforming growth factor-alpha and epidermal growth factor, trefoil peptides, and/or other regenerating proteins, could be potential candidates responsible for the higher level of mucus secretion observed in the LDA-takers of this study because these molecules are known to act as protective agents for the gastric epithelium and to be induced by the administration of aspirin or other NSAIDs [37–39].

In conclusion, our results suggest the potential contribution of a reactive increase in gastric mucus secretion to the gastric adaptive response to repeated doses of LDA. Therapeutically, these results also suggest that, in addition to the inhibition of gastric acid secretion, the potentiation of gastric mucus secretion could be another promising approach for the prevention of LDA-induced gastropathy.

Conflict of interest None.

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