Carcinogenic potential of duodenal reflux juice from patients with long-standing postgastrectomy

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Abstract

AIM To determine whether study on the carcinogenic potential of reflux juice from patients with remote gastrectomy could clarify the inherent relationship between duodenal reflux and gastric stump cancer.

METHODS A total of 37 reflux juice samples (13 BillrothI, 24 BillrothII) were employed in the present study. A two-stage transformation assay using BALB/c 3T3 cells was carried out to test the initiating or promoting activity of these samples.

RESULTS Two of 18 (11.1%) reflux samples exerted initiating activities, whereas 9/19 (47.4%) samples enhanced the MNNG-initiating cell transformation, suggesting the duodenal reflux juice might more frequently possess the tumor-promoter activity (P = 0.029). In addition, there was no difference in initiating activities of the samples irrespective of surgical procedures (P = 0.488), while Billroth II samples exhibited stronger tumor-promoter activity than Billroth I samples (P = 0.027). Furthermore, the promoter activities were well correlated with the histological changes of the stomas (rₛ = 0.625, P = 0.004), but neither their cytotoxicities nor initiating activities had this correlation (Probabilities were 0.523 and 0.085, respectively).

CONCLUSION The duodenal reflux juice from patients with remote postgastrectomy did have carcinogenic potential, and suggested that tumor-promoting activity should principally account for the high incidence of gastric cancer in gastrectomy patients. In contrast, it is difficult to explain the high stump-cancer incidence with the “N-nitroso compounds” theory—a popular theory for the intact stomach carcinogenesis, and it seemed to be justified to focus chemoprevention of this cancer on the tumor-promoting potential of reflux juice.

INTRODUCTION

Since gastric stump cancer was first described in 1922¹, it has been well established that the incidence of gastric carcinoma is increased in patients who have undergone a partial gastrectomy for peptic ulcer disease²-⁶. But the etiology and exact mechanism of gastric stump carcinogenesis are unclear. Decreased sensitivity of chief cells and parietal cells⁷, alteration in gastrin level⁸, hypoxia and hemodynamic changes⁹, bacterial proliferation¹⁰, and reflux¹¹,¹² are the putative contributing factors. Among these, the excessive duodenal reflux induced by surgery seems to be the main risk factor, because the incidence of stump carcinomas is higher in Billroth II than in Billroth I¹³,¹⁴, and most of the stump carcinomas are located near the stoma¹⁵-¹⁷. Nevertheless, what is the inherent association between the duodenal reflux and stump cancer? To answer the question, a cell transformation assay was employed in our study to examine the carcinogenic potential, initiating and promoting activity, of reflux juices from patients with remote postgastrectomy, in terms of the common theory carcinogenesis is analytically considered to relate to the two stages, initiation and promotion¹⁸-²³.

MATERIALS AND METHODS

Sampling

Thirty-seven patients (10 women and 27 men; aged, 42-77 years) who underwent partial gastrectomy at least 10 years previously for benign ulcer disease received endoscopy. Each patient experienced either BillrothI or BillrothII procedures (Table 1). Endoscopy was carried out after 8 to 12 hours of fasting. The bile stained reflux fluid was aspirated in a sterile syringe, then sterilized by passing through a 0.22 µm Millipore filter and stored at -70°C until analysis. The thirty seven patients were all eligible for this study (patients with histories of smoking were excluded from the study), and informed consent was obtained from each patient.
For the sake of enough reflux juice, samples from Billroth or Billroth II patients were randomly extracted to make two almost equal sized groups to evaluate the initiating and promoting activity, respectively. At the end of the endoscopy, at least five biopsies in a circle around stoma were taken for histological assessment. Three grades were used which were, in ascending order of significance: chronic superficial gastritis (± minimal atrophic gastritis); atrophic gastritis/intestinal metaplasia; dysplasia[24].

Cytotoxicity assay
BALB/c 3T3 A31-1-1 cells 1 × 10⁴ (one of three standardized cell lines generally recommended for the cell transformation assay)[25] were plated in each well of a 96-well plate covered with 100 µL DMEM (Dulbecco’s modification of Eagle’s medium, Gibco) supplemented with 10% FCS (fetal calf serum, Gibco) at 37°C in a humidified incubator containing 5% CO₂ in air for 24 hours. The medium was then replaced by 100 µL medium containing reflux juice (15-20 doses designed serially per sample by a concentration gradient of 1.25% reflux juice), and further incubated for 24 hours. The culture was used as a negative control; 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT; Promega) was added (5 g/L) and the plates were incubated for a further 4 hours. The dye/medium in each well was carefully removed and 100 µL solubilization solution (Promega) was placed in each well for 1 hour. The plates were read at 570 nm in a microplate reader (Biorad 550). The mean absorbance was calculated and cell survival was expressed as the percentage absorbance of that in wells incubated with the negative control.

Transformation assay
Two-stage transformation was assayed by the protocol described by Hirakawa et al[26]. Only the volume of culture medium was changed from 5 mL to 4 mL. In the initial assay, actively growing cells (10⁴ cells per 60 mm-diameter plastic dish) were plated. Cultures were incubated for 24 hours, reflux samples with graded concentrations (80% and 40% critical toxicity) were added for 72 hours (initiating phase), and 0.3 mg/L TPA (12-O-tetradecanoylphorbol 13-acetate) were present in the medium for 2 weeks 4 days after reflux juice was removed (promoting phase). The medium was then replaced with fresh, promoter-free medium and the culture was incubated for another 2 weeks. The culture medium was changed twice a week. Ten dishes were used for each sample in 2 independent tests. MNNG (N-methyl-N’-nitro-N-nitro-sooguanidine) 0.5 mg/L and culture medium were used as positive and negative control, respectively. Finally, the culture cells were fixed and stained with Giemsa 5 weeks after plating. Type III transformed cell foci (deeply basophilic, criss-crossing, a dense layer formation and a random orientation of cells at the edges of foci[25]) were counted. As for the transformation frequency (TF), the percentage of dishes with foci was calculated. In the promoting assay, 0.5 mg/L MNNG (dissolved in DMSO) was employed in the initiating phase, and reflux samples (25% toxicity) were added into the medium during the promoting phase. Twelve dishes were used for each sample in 2 independent tests. TPA 0.3 mg/L and culture medium were taken as positive and negative control, respectively. Other procedures of this assay were the same as described above.

Statistical analysis
One-way ANOVA was used to search for differences in the average TF values between Billroth I or Billroth II groups. In other sections, two-tailed Fisher’s exact test was performed. A probability of P<0.05 was considered statistically significant and we used Bonferroni’s method to get the nominal level for each comparison of TF between a sample and the culture control.

RESULTS
In the 2-stage transformation assay, without a known promoter TPA, a 3-day initiating treatment with a potent carcinogen MNNG at 0.5 mg/L had not significantly yielded an increasing number of transformed foci. While MNNG caused a very remarkable transformation with subsequent TPA (0.3 mg/L) promoting treatment (data not shown). The results suggested that the present assay system work well enough to examine whether an agent could exert an initiating or promoting activity. In addition, the doses of bile samples used in the cell transformation assay were selected based on the results of MTT (toxicity) assay. A small concentration gradient (1.25% reflux juice, v:v) was used in MTT assay to obtain the critical cytotoxicity more accurately. Because the toxicity of the samples were different (ranging from 75 to 250 µL aspirate/mL medium), a unified criterion (the same percent of each sample’s own toxicity) was used to compare TF values among various samples effectively. In the initiating assay, the aspirate concentration was extended to a high dose, 80% critical toxicity, to ensure that our conclusions are free from false negative results. A low dose, 25% toxicity, was used in the promoting assay for the sake of the culture cells having to survive a long-term exposure to reflux juice (2 weeks). In addition, this dose-design method makes it feasible to study on the carcinogenic potential independent of the cytotoxicity of reflux juice.

Carcinogenic potential in the transformation assay
Six Billroth I and 12 Billroth II samples were randomly distributed into the initiating group. Two of 18 samples exerted significantly initiating activity compared with negative control group in the TPA-promoting cell transformation assay (Figure 1a). As
analysed by subsite, 1/6 Billroth I and 1/11 Billroth II samples were positive. There was no marked difference in the average TF values between the two groups (Figure 1b) either at the high or low doses (data not shown for the low dose, 40% toxicity).

The residual samples, 7 Billroth I and 12 Billroth II, made up the promoting group. As shown in Figure 2a, there was an increased number of transformed foci in the MNNG-initiated target cells followed by applications of 9 of 19 samples. The nine positive samples included 2 Billroth I and 7 Billroth II aspirate. A significant difference in the average TF values was observed between the two groups (P<0.05, Figure 2b).

In comparison of the results between the initiating and promoting assay, the promoting positive rate (9/19, 47.4%) was statistically higher than initiating positive rate (2/18, 11.1%) (P = 0.029). Therefore, the reflux juice might usually exhibit the promoting activity and sparsely possess the promoting potential.

Histological grades with carcinogenic potential and cytotoxicity of reflux juice

A stronger correlation (Spearman’s rank correlation coefficient r = 0.625, P = 0.004) was found between the histological grades of stoma and the promoting activity, but not between histological grades and the initiating activity (Table 2). In addition, with the upward shift of histological grades, a small decrease in the toxic concentrations of reflux juice was observed in Table 1, which did not reach statistical significance.

**Table 1** Operation types and critical toxicity of reflux juice in each histological grade

| Histological grades                      | Billroth I | Billroth II | Critical toxic concentrations (% v:v) | Correlation between histological grades and toxicity* |
|------------------------------------------|------------|-------------|--------------------------------------|-----------------------------------------------------|
| 1 Chronic superficial gastritis          | 7          | 10          | 16.4±4.0                              | r_s = -0.287, P = 0.085                              |
| 2 Atrophic gastritis/intestinal metaplasia | 4          | 7           | 15.8±5.0                              |                                                     |
| 3 Dysplasia                              | 2          | 7           | 12.9±4.6                              |                                                     |

*Two tailed Spearman’s rank correlation.

**Table 2** Histological grades of stoma and carcinogenic potential of reflux juice in the cell transformation assay

| Histological grades                      | Initiating (%) | Promoting (%) | Correlation coefficient* |
|------------------------------------------|----------------|---------------|--------------------------|
| 1 Chronic superficial gastritis          | 20.1±18.4 (8)  | 25.3±18.7 (9) | r_s = 0.161              |
| 2 Atrophic gastritis/intestinal metaplasia | 26.0±20.7 (5) | 48.1±20.1 (6) | r_s = 0.625              |
| 3 Dysplasia                              | 20.4±7.10 (5) | 56.7±10.6 (4) | P = 0.523                |

*Two tailed Spearman’s rank correlation. The number of the patients for each histological grade is inside the parentheses.
DISCUSSION

In present study, the duodenal reflux juice from patients with remote postgastrectomy exerted more frequently tumor-promoter activity compared to the initiating (mutagenic) activity. In addition, there was no difference in the initiating activity of reflux samples irrespective of surgical procedures. While the BillrothII aspirates exhibited a stronger tumor-promoter activity than BillrothI, in accordance with many epidemiological findings, stump cancer preferred to the BillrothII procedure. All these results strongly suggested that partial gastrectomy - perse- should be responsible for the reflux promoting activity, implying an etiological role for the promoting activity of duodenal reflux juice in the pathogenesis of gastric stump cancer. It is just the tumor-promoter activity, a characteristic beyond what an intact stomach usually possesses, that may elucidate the high incidence of gastric cancer in postgastrectomy patients than in general population.

As for the etiology of gastric cancer, dietary factor has been emphasized principally since some procarcinogens, such as nitrates, can often enter the diet by vegetables, preservatives of food, even drinking water. Nitrite may derive from nitrate by the flora of the mouth or stomach (human saliva typically contains 6-10 mg/L nitrite and 15-35 mg/L nitrate[27]), and further react with secondary amines to give rise to N-nitroso compounds which are strong carcinogens suspected of playing a role in upper gastrointestinal carcinogenesis[28] because of their spontaneous synthesis from dietary components and their ability to alkylate nucleic acids. This “N-nitroso compounds” theory has provided a potential explanation for some geographic regions at a very high risk for gastric cancer[29]. But whether this theory can work as well to interpret the high incidence of gastric cancer in the postgastrectomy patients is still a matter of dispute[30-34]. Although we did not measure the concentrations of N-nitroso compounds directly, the initiating assay can effectivly detect the mutagenicity of the whole human reflux juice that may contain various mutagenic or carcinogenic substances inclusive of the N-nitroso compounds. When analysing our data from another angle, the initiating or mutagenic activity of reflux samples did not correlate to the histological grades of anastomotic area (Table 2), whereas the promoting activity significantly augmented with the progression of histological abnormalities. Thus, a causal role for the promoting activity of reflux juice in the pathogenesis of stump cancer was further suggested, implying that the tumor-promoter activity might principally account for the high incidence of gastric cancer in the long-standing postgastrectomy patients relative to the initiating activity. While this did not, of course, exclude that the initiating activity or mutagenic activity was indispensable to the stump carcinogenesis. But a perfect explanation for the high stump-cancer incidence seemed to be not available by the “N-nitroso compounds” theory, as it is different from the setting of intact stomach carcinoma. Rising gastric pH in the presence of bacteria after gastrectomy might not favor the formation of the mutagenic or carcinogenic compounds (e.g., N-nitroso compounds)[14,35], and those with a high concentration enough to take an initiating or mutagenic effect were seldom present in the reflux juice of postgastrectomy patients. In addition, as shown in Table 1, with the histological manifestations of the stoma, a slight increase in the non-specific cytotoxicity of reflux juice was observed. Although it did not reach the statistical significance, whether the result alluded to a synergic effect for the reflux toxicity in the gastric stump carcinogenesis deserved further studies.

What is the exact nature of the tumor-promoting species in the reflux juice Some substances exerting a persistent action (e.g., cell differentiating or proliferating[36-39]) must exist in the reflux juice. Unconjugated and secondary bile acids might be first candidates, which have been suggested to take a part in the colonic cancer[40-46]. These bacteria-degraded bile acids also present in the gastric aspirate from remote postgastrectomy patients though consisting of a small portion of the whole reflux bile acids. Secondly, lysophosphatidylcholine (lysoPC), the product of phosphatidylcholine hydrolysis by phospholipase A2, has been suggested to play a role in the pathogenesis of gallbladder cancer in the APBDJ (Anomalous Pancreaticobiliary Ductal Junction) patients[47]. LysoPC might also be produced in the stump stomach due to the reflux juice containing aberrant pancreatic juice and bile. It has been reported that lysoPC at much lower concentrations significantly enhanced the activation of protein kinase C (PKC)[48-50] and regulated cell differentiation[51] if diacylglycerol (DAG) was available. And the unconjugated bile acids happened to remarkably generate DAG[52]. Therefore, whether the above two factors could cooperate to make the reflux juice exhibit tumor-promoter activity This issue also warrants further investigations.

For the first time, we confirmed that the duodenal reflux juice from the long-standing postgastrectomy patients did exert the tumor-promoting activity and initiating activity, and further suggested that tumor-promoting potential should be mainly responsible for the high stump-cancer incidence. Simultaneously, our results demonstrated that a close relationship between the histological changes of anastomotic site and the reflux tumor-promoting activity, thus directly supporting to conduct endoscopic surveillance for postgastrectomy patients with precancerous lesions.
(e.g., moderate and severe dysplasia) in stoma. In addition, to decrease the incidence of stump cancer effectively, it seems reasonable not only to perform reconstruction procedures (e.g., Roux en Y anastomosis) for those with severe duodenal reflux, but also to focus the chemoprevention of this cancer on tumor-promoting potential of the reflux juice.

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