Retinoic acid formation from retinol in the human gastric mucosa: role of class IV alcohol dehydrogenase and its relevance to morphological changes

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Submitted 9 November 2004; accepted in final form 26 April 2005

Alcohol dehydrogenase (ADH) participates in the formation of retinoic acid from retinol in various organs including the gastric mucosa. However, its clinical significance still remains to be clarified. In this study, we identified the ADH isoforms responsible for the retinoic acid formation among various ADH isoforms and examined associations among the ADH activities, the retinoic acid formation level, and morphological changes in the human gastric mucosa. Human gastric samples were endoscopically obtained from 67 male subjects. Morphological changes were assessed by the Sydney system and activities of class I, III, and IV ADH isoforms were determined in each specimen. In 26 cases, levels of all-trans retinoic acid (ATRA) formation from all-trans retinol were examined. Among activities of the three ADH isoforms, class IV ADH activity was solely associated with the ATRA formation level. This association was found even when subjects’ age and Helicobacter pylori infection status were adjusted. As the degrees of inflammation, atrophy, and intestinal metaplasia increased, the class IV ADH activity was well as the potential for the ATRA formation decreased. Class IV ADH is a major enzyme in the retinoic acid formation in the human gastric mucosa, and the reduction of its activity was associated with decreasing retinoic acid supply and progression of inflammation, atrophy, and intestinal metaplasia in the gastric mucosa. In that retinoic acid is a key molecule for maintaining normal morphology, the reduction of class IV ADH activity may be involved in the pathogenesis of these morphological changes in the human gastric mucosa.

σ-alcohol dehydrogenase; vitamin A; atrophy; intestinal metaplasia; Helicobacter pylori

Retinoic acid is formed from retinol (vitamin A) via a two-step oxidation process (7) involving various enzymes including some forms of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). The former oxidizes retinol (an alcohol) to retinal (an aldehyde) and the latter oxidizes retinal to retinoic acid. We have demonstrated that the human gastric mucosa has such an oxidation pathway, and at least some ADH isoforms are responsible for the pathway because several ADH inhibitors hampered it (36). However, three ADH forms, namely class I (γ), class III (χ), and class IV (σ) ADHs, are expressed in the human gastric mucosa (22, 33), and it is still obscure which isoform is responsible for the retinoic acid formation from retinol. The first aim of this study was to solve this question. A method for the quantification of retinoic acid that was recently established (35) and improved in our laboratory (19) enabled this study.

Retinoic acid is known as an essential molecule for maintaining normal proliferation and differentiation of cells in various organs (9) including the stomach. Accordingly, several groups have proposed that regional ADHs should play a significant role in morphology of the gastric mucosa via retinoic acid supply (10, 30, 34). However, there exists no direct evidence supporting the view to this day. The second aim of this study was to verify the rationality of the proposals. To that effect, the relevance among morphological changes, the ADH activities, and the potential for all-trans retinoic acid (ATRA) formation from all-trans retinol was examined in the human gastric mucosa.

MATERIALS AND METHODS

Materials and human gastric mucosa samples. Most chemicals and supplies were purchased from Sigma-Aldrich (St. Louis, MO). All studies involved in this report were approved by the Ethics Committee of School of Medicine, Keio University, based on the ethics guidelines of the 1975 Declaration of Helsinki. Male patients undergoing endoscopy examinations of upper digestive tracts for various reasons were asked by written consent form to provide extra tissues from the midcorpus region of the stomach for research purposes. Finally, samples were collected from 67 donors aged 50.3 ± 13.7 (mean ± SD) years who understood the aims of the study and agreed to donate samples. After sampling, half of each specimen was fixed in 10% formaldehyde for histological examination and the remaining tissue was quickly frozen in an Eppendorf tube by liquid nitrogen and stored at −80°C until assayed as follows. From the latter parts, the cytosolic fractions were prepared as described in previous reports (17) just before the experiments. The amount of protein in each fraction was measured by Lowry’s method (16) using a kit. Among the 67 cases, extra samples were available in 26 cases and the formation of retinoic acid from retinol could be further examined in them.

Activity of classes I, III, and IV ADHs in the human gastric mucosa. Activities of the human gastric ADHs were assessed according to the methods previously reported (6, 17). Activities of class I and IV ADHs were measured by the reduction of their preferred substrates, namely acetaldehyde and m-nitrobenzaldehyde, respectively, in the presence of NADH. Briefly, 200 µg of each human gastric cytosolic protein was incubated at 37°C in 0.1 M sodium phosphate buffer (pH 7.5) containing 100 µM of NADH and 250 µM of acetaldehyde or 100 µM of m-nitrobenzaldehyde, with a total volume of 3 ml. Decrease in absorbance at 340 nm was measured for 5 min using a spectrophotometer (model MPS-200; Shimadzu, Tokyo, Japan). The activity of class III ADH was measured by the oxidation of its preferred substrate, formaldehyde, in the presence of GSH and NAD. Briefly, each sample was incubated at 25°C in 0.1 M sodium pyro-
phosphate buffer (pH 8.0) containing 1.2 mM of β-NAD, 1 mM of GSH, and 1 mM of formaldehyde (Wako Junyaku, Tokyo, Japan), with a total volume of 3 ml. Evaporating of substrates during the incubation was prevented by tightly covering each cuvette with Parafilm. A mixture without gastric sample was used as the blank of each spectrophotometrical ADH assay. Increase in absorbance at 340 nm was measured for 5 min using the spectrophotometer. Class I and IV ADH activities were expressed in nanomoles NADH oxidation per minute per milligram protein and that of class III ADH was done in nanomoles NAD reduction per minute per milligram protein.

Formation of ATRA from all-trans retinol. The level of ATRA formation from all-trans retinol was assessed in 26 cases based on the HPLC methods established in our laboratory (19, 35). Five hundred micrograms of each human gastric cytosolic fraction were incubated with 3 μg of all-trans retinol and 2 mM of β-NAD at 37°C for 20 min in 20 mM of HEPES buffer (pH 7.5) containing 150 mM of potassium chloride and 2 mM of β-mercaptoethanol, in a total volume of 1.5 ml. After the reaction, 500 μl of ethanol, 500 μl of saturated ammonium sulfate, 2 ml of distilled water, and 4 ml of a solution containing n-hexane, acetic acid, and dichloromethane in a ratio of 80:9:1 were added to 500 μl of each reaction mixture and shaken for 10 min. Twenty micromoles of arotinoid ethylsulfone, kindly donated by Hoffmann-La Roche (Basel, Switzerland), were added to each sample as an internal standard. Samples were centrifuged at 2,700 g for 4 min, and the organic layer (upper layer) was collected using a glass pipette. The organic solvent was evaporated using nitrogen gas, and the extracts were resolved in 200 μl of n-hexane. Fifty microliters of the solution were used for HPLC quantification, by the method established by our laboratory (19). Areas under the curve for ATRA and arotinoid ethylsulfone used as an internal standard were calculated by a chromatointegrator. The ATRA level in each sample was quantified by using a working curve established in our laboratory, adjusting the level of the internal standard (19, 35). All procedures were performed under a safety light or in the dark to avoid photoisomerization of the retinoids (35).

Histological assessment. Three-micrometer-thin sections were prepared from the paraffin-embedded specimens and stained with hema-toxylin and eosin. Specimens were examined under a light microscope by an expert in stomach pathology, and the degree of each parameter was ranked according to the Sydney system (25). Statuses of inflammation, atrophy, intestinal metaplasia, and neutrophil infiltration were evaluated using a graded scale: absent (grade 1), mild (grade 2), moderate (grade 3), and severe (grade 4). Degree of neutrophil infiltration was not taken into further consideration in this study because all cases were classified as grade 4. The presence of Helicobacter pylori infection in situ was also judged in each hematoxylin and eosin-stained specimen.

Statistical analysis. Values were described as means ± SD or SE. The difference between multiple groups was assessed by one-way ANOVA with Fisher’s exact test as a post hoc test. The correlations among multiple groups were assessed by multiple regression analysis. A P value of <0.05 was considered statistically significant.

### Table 1. Results of morphological analyses of the human gastric mucosa based on the Sydney system

<table>
<thead>
<tr>
<th>Grade</th>
<th>Inflammation</th>
<th>Intestinal Metaplasia</th>
<th>Atrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>42</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>6</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td>67</td>
<td>67</td>
</tr>
</tbody>
</table>

Values are expressed as numbers of subjects. Degree of morphological analyses were evaluated using a graded scale: absent (grade 1), mild (grade 2), moderate (grade 3), and severe (grade 4).

### Table 2. Association between ADH activities and levels of ATRA formation in the human gastric mucosa

<table>
<thead>
<tr>
<th>Class</th>
<th>Regression Coefficient ± SE</th>
<th>Standardized Regression Coefficient</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td>0.114 ± 0.094</td>
<td>0.210</td>
<td>0.2451</td>
</tr>
<tr>
<td>Class III</td>
<td>0.705 ± 0.365</td>
<td>0.329</td>
<td>0.0730</td>
</tr>
<tr>
<td>Class IV</td>
<td>0.749 ± 0.199</td>
<td>0.642</td>
<td>0.0019</td>
</tr>
</tbody>
</table>

Values are multiple regression analysis for levels of retinoic acid formation; n = 26 subjects. Multiple coefficient of determination (R^2), 0.580; adjusted multiple coefficient of determination, 0.490; ADH, alcohol dehydrogenase; ATRA, all-trans retinoic acid.

### RESULTS

The activities of class I, III, and IV ADHs were 16.1 ± 11.1 NADH oxidation·min⁻¹·mg protein⁻¹, 2.7 ± 1.7 NAD reduction·min⁻¹·mg protein⁻¹, and 9.4 ± 4.4 NADH oxidation·min⁻¹·mg protein⁻¹, respectively, in this population. Results of histological examinations in 67 gastric specimens according to the Sydney system (25) are summarized in Table 1.

Among the 67 subjects, ATRA formation levels from all-trans retinol in the human gastric mucosa were studied in 26 samples and the correlation between the ATRA formation level and activities of the three ADH forms was studied by multiple regression analysis. As shown in Table 2, the activity of class IV ADH was correlated with the ATRA formation (P = 0.0019), whereas those of class I (P = 0.2451) and III (P = 0.0730) ADHs were not. The association between class IV ADH activity and the retinoic acid formation level was further confirmed by multiple regression analysis in which subject age and in situ H. pylori infection status were adjusted (P = 0.0020; Table 3).

The relationships between three ADH activities and morphological changes in the gastric mucosa as judged by the Sydney system were then also examined by multiple regression analysis. As shown in Table 4, degrees of inflammation, intestinal metaplasia, and atrophy were inversely correlated with class IV ADH activities in the human gastric mucosa, but not with those of class I and III ADH activities. The inverse correlations between class IV ADH activity and the degrees of the three parameters of morphological changes were confirmed by multiple regression analysis in which subject age and in situ H. pylori infection status were adjusted (Table 5).

The associations between the morphological changes and the level of retinoic acid formation were then studied in the gastric specimens. As shown in Fig. 1, as degrees of inflammation (λ = 22.72, P = 0.0003, Fig. 1A), intestinal metaplasia (λ = 8.12, P = 0.0687, Fig. 1B), and atrophy (λ = 10.80, P = 0.0116, Fig. 1C) increased, the retinoic acid formation levels significantly decreased or showed a trend to decrease.

### DISCUSSION

In the present study, a key enzyme for ATRA formation from retinol in the human gastric mucosa was identified. Furthermore, the relationships among ADH activities, potential for ATRA formation from all-trans retinol, and features of histological changes were examined in the human gastric mucosa. Recently, a gender difference in gastric ADH activities has been reported (1). Although the view seems to be still
equivocal (15, 17, 20, 23), male subjects were selectively studied at this time. In this study, each ADH isoform was spectrophotometrically evaluated using its preferred substrate (6). Although slight errors including that due to the evaporation of substrate and that due to types of blanks are inevitable in a spectrophotometrical assay, methods used in the present study are widely accepted (4, 6). In this study, a specimen without a gastric sample was used as a blank for each spectrophotometrical assay. Although other types of blanks including ones without substrate or without coenzyme could be applicable, we chose our method because of the limitation of sample availability.

Our previous findings that several ADH inhibitors hampered this NAD-dependent ATRA formation from retinol in the human gastric mucosa (36) suggested that some ADHs have a significant role in this process, at least in part. In the present study, we clarified which ADH form is responsible for this process among three ADH isoforms expressed in the human gastric mucosa. The present multiple regression analysis showed that only class IV ADH but not class I and III ADHs were associated with the ATRA formation level. This indicates that among ADH isoforms in the human gastric mucosa class IV ADH has a major role on the NAD-dependent ATRA formation. Compared with the $K_m$ values for retinol of three ADHs expressed in the human gastric mucosa, i.e., class I, class III, and class IV ADHs, the finding is reasonable (3, 11, 14). The association between the class IV ADH activity and the ATRA formation level was further confirmed even after adjusting subject age and status of $H. pylori$ infection in another multiple regression analysis. Although factors different from ADH, including ALDH activity and substrate concentration should also affect this process, we could not examine these points at this time because of the limitation of sample availability.

In the present study, the ATRA formation in the human gastric mucosa was studied in the protocol established in our laboratory (36). Because the concentration of all-trans retinol in the protocol (0.007 mM) was similar to the reported $K_m$ value for all-trans retinol of class IV ADH (0.008–0.0031 mM; Refs. 3, 11, and 14), it might not provide its $V_{max}$, i.e., the retinol oxidation rate measured here would be different from optimal one. Because all specimens were studied in the equal conditions, the comparison of the ATRA formation level in this study should be meaningful even if the enzyme activity is not maximal. However, we must be careful for the interpretation of the present findings.

Although the associations between the ADH activities and histological changes in the gastric mucosa have already been demonstrated (8, 20, 27), the advantages of the present study were that 1) activities of three ADH isoforms were specifically and separately assessed using their preferred substrates and 2) histological changes in the gastric mucosa were systematically assessed by the Sydney systems (25). Seitz et al. (27) reported an inverse association between the degree of inflammation and the ADH activity in the gastric mucosa. Consistently, the present multiple regression analysis showed an inverse association between the class IV ADH activity and the development of inflammation in the human gastric mucosa. Simanowski et al. (28) suggested that development of inflammation causes a loss of normal cells expressing ADH and that this can explain why the ADH activities are reduced in the gastric mucosa with inflammation.

In the present study, we further demonstrated that progression of atrophy and intestinal metaplasia were also associated with decreasing class IV ADH activity in the human gastric mucosa even after adjusting subject age and $H. pylori$ infection status. Because loss of normal cells should occur also in gastric mucosa with atrophy or intestinal metaplasia, the reduction of class IV ADH activity in these specimens may be also just secondary to pathologic changes as mentioned by Simanowski et al. (28).

It was interesting to observe in the present study that the progressions of these morphological changes were inversely associated with the ATRA formation level in the human gastric mucosa. In that the class IV ADH is a key enzyme for the retinoic acid formation from retinol and its activity was inversely associated with the progression of inflammation, atrophy, or intestinal metaplasia, the findings per se were fully expected. However, it is notable that retinoic acid is an essential molecule for various cells to maintain their normal proliferation and differentiation (9). Moreover, the reduction of retinoic acid signal has been implicated in the development of premalignant lesions in the human gastric mucosa such as intestinal metaplasia and atrophy (12). Although retinoic acid should be systematically supplied to each cell, the significance of the regional retinoic acid supply system has recently been proposed (10, 30, 34). Taken together, contrary to the view that the pathologic changes result in the reduction of ADH activity, there is a possibility that the reduction of class IV ADH activity may cause pathologic changes in the gastric mucosa.

The current consensus is that $H. pylori$ infection plays a significant role in the pathogenesis of the human gastric mucosa.

<table>
<thead>
<tr>
<th>Regression Coefficient ± SE</th>
<th>Standardized Regression</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.006±0.007</td>
<td>0.114</td>
</tr>
<tr>
<td>$H. pylori$ infection status</td>
<td>0.106±0.200</td>
<td>0.072</td>
</tr>
<tr>
<td>Class IV ADH activity</td>
<td>0.075±0.023</td>
<td>0.452</td>
</tr>
</tbody>
</table>

Values are multiple regression analysis for levels of retinoic acid formation; $n = 26$ subjects. Multiple coefficient of determination ($R^2$), 0.254; adjusted multiple coefficient of determination, 0.203.

Table 4. Association between ADH activities and morphological changes in the human gastric mucosa

<table>
<thead>
<tr>
<th></th>
<th>Inflammation</th>
<th>Intestinal metaplasia</th>
<th>Atrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I ADH activity</td>
<td>−0.092 (0.6176)</td>
<td>−0.097 (0.7890)</td>
<td>−0.033 (0.9108)</td>
</tr>
<tr>
<td>Class III ADH activity</td>
<td>−0.096 (0.7070)</td>
<td>−0.075 (0.8349)</td>
<td>0.222 (0.4555)</td>
</tr>
<tr>
<td>Class IV ADH activity</td>
<td>−0.817 (0.0025)</td>
<td>−0.553 (0.0097)</td>
<td>−0.759 (0.0365)</td>
</tr>
</tbody>
</table>

Compared with three independent variables* 0.804 (0.731) 0.257 (0.193) 0.495 (0.306)

Values are multiple regression analysis for levels of retinoic acid formation; numbers in parentheses are $P$ values; $n = 67$ subjects. SRC, standardized regression coefficient. *Multiple coefficient of determination (adjusted multiple coefficient of determination).
closa (21). Interestingly, H. pylori infection has been reported to be associated with decreased ADH activity in the human gastric mucosa (4, 13, 17, 29). In view of our present findings, decrease of class IV ADH and the subsequent disturbance of retinoic acid supply may, at least in part, be involved in the mechanism by which H. pylori infection causes pathologic changes in the human gastric mucosa. The recently prevailing view is that H. pylori carries ADH and it may have various pathogenic roles (26). It is, therefore, postulated that exogenous H. pylori ADH may affect retinol metabolism in human gastric mucosa. However, it is most likely that the role of the H. pylori ADH on retinol oxidation is negligible in the human gastric mucosa because gastric mucosa with H. pylori infection have lower ADH activities as well as lower potential of retinol oxidation than that without it.

From the present findings, a scenario in which the sequence of the cell loss due to inflammation, the reduction of class IV ADH, and the disturbance of retinoic acid supply results in the development of various morphological changes in the gastric mucosa could be drawn. This is consistent with Correa cascade theory referring to the sequential development of various lesions in the gastric mucosa (5). The endpoint of the Correa cascade is the development of malignancy (5). Coincidently, insufficiency of retinoic acid supply also causes malignancies ultimately in various organs (31, 32), although it is still unclear that it causes gastric cancer.

The activity of class IV ADH was solely associated with the morphological changes in the human gastric mucosa among three gastric ADH isoforms. Similarly, Chrostek et al. (4) reported that H. pylori infection causes the exclusive reduction of class IV ADH activity in the human gastric mucosa. Differences in the localization of three ADH isoforms in the gastric mucosa (10) may, at least in part, account for the dissociation of effects of morphological changes on class IV ADH and those on the other ADHs. It is conceivable that the impact of cell loss is expected to be, at least, smaller on class III ADH compared with the other ADHs because class III ADH is broadly expressed in the gastric mucosa (10), whereas the other ADHs are localized in limited loci (10, 24). In addition, if the reduction of class IV ADH activity causes the morphological changes in the human gastric mucosa, this also could account for the exclusive association between the class IV ADH and the morphological changes in the human gastric mucosa.

Amounts of retinol, retinal, and retinoic acid in situ in relation to activities of ADH and ALDH should be quantified in order to understand the mechanisms and significance of the local retinoic acid supply system in organisms. However, their amounts contained in the specimens prepared from endoscopically obtained samples were too small to quantify by our HPLC methods. Recent progress in techniques including the

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**Table 5. Association between class IV ADH activity and morphological changes in the human gastric mucosa**

<table>
<thead>
<tr>
<th>SRC</th>
<th>Inflammation</th>
<th>Intestinal metaplasia</th>
<th>Atrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.146 (0.2083)</td>
<td>0.104 (0.5062)</td>
<td>0.114 (0.3931)</td>
</tr>
<tr>
<td>H. pylori infection status</td>
<td>0.296 (0.0221)</td>
<td>0.283 (0.0352)</td>
<td>0.221 (0.0725)</td>
</tr>
<tr>
<td>Class IV ADH activity</td>
<td>-0.582 (&lt;0.0001)</td>
<td>-0.428 (0.0142)</td>
<td>-0.452 (0.0020)</td>
</tr>
<tr>
<td>Compared with three independent variables*</td>
<td>0.439 (0.401)</td>
<td>0.217 (0.146)</td>
<td>0.254 (0.203)</td>
</tr>
</tbody>
</table>

Values are multiple regression analysis for levels of retinoic acid formation; numbers in parentheses are P values; n = 67 subjects. *Multiple coefficient of determination (adjusted multiple coefficient of determination).
establishment of HPLC-MS (2, 18) may be applicable to detect these retinoids in situ.

In conclusion, among three gastric ADHs, class IV ADH is prominent in the production of retinoic acid from retinol. The progression of morphological changes such as inflammation, atrophy, and intestinal metaplasia in the gastric mucosa were associated with the reduction of class IV ADH activity as well as the decrease in retinoic acid supply. In that retinoic acid is a significant substance for maintaining normal morphology in various organs and the reduction of retinoic acid is implicated in the pathogenesis of premalignant lesions in the gastric mucosa, the reduction of class IV ADH activity may be a significant event leading to the development of morphological changes in the human gastric mucosa.

REFERENCES


