Comparison of the Distribution of Carbonic Anhydrase Isozymes (CA-I, CA-II, CA-III) in the Rat Gastrointestinal Tract

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ABSTRACT. The present paper described the immunohistochemical distributions of carbonic anhydrase (CA) isozymes, CA-I, CA-II and CA-III, in the epithelium lining the rat gastrointestinal tract, with rabbit antibodies to equine CA-I, CA-II and CA-III. Prior to the immunohistochemical examinations, the crossreactivities of these antibodies to the rat-antigens were confirmed in this study. In the stomach, surface epithelial cells and parietal cells of the glandular region showed an immunoreactivity only to CA-II. In the large intestine, each immunoreactivity to CA isozyme (CA-I, CA-II and CA-III) was localized in the upper portion of intestinal glands, and decreased toward the distal digestive tract, but absent in the small intestine. The present histological findings suggested that the CA isozymes might play a role in the ion-transportation during the water absorption in the rat large intestine.—**KEY WORDS:** carbonic anhydrase, gastrointestinal tract, immunohistochemistry, rat.

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Carbonic anhydrase (CA) catalyzes hydration of CO₂ and dehydration of H₂CO₃ ($\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3^- + \text{H}^+$), which can occur at extremely high rates. Since CA is one of the most important enzymes, most living things such as some bacteria [23], plants [24], and animals [7, 9, 13, 19, 24] must have some types of CA isozymes. The previous investigations in the biochemistry and physiology have gradually thrown light upon the role of CA isozymes in the biological functions. On the other hand, the precise histolocalization of CA isozymes is essential to explain some actions of the isozymes in the organ or tissue.

Recently, Spicer *et al.* [20] described the expressions of CA-II and CA-III in the large intestine of the mouse by immunohistochemical methods with anti-human CA-II and CA-III sera. However, it is very important to know the more precise distributions of CA isozymes in the gastrointestinal tract of several species for the understanding of functions of CA isozymes. In the present paper, therefore, the immunohistochemical localization of CA isozymes in the rat stomach and large intestine have been described by the use of the antibodies to CA-I, CA-II and CA-III.

MATERIALS AND METHODS

Five males and females of each of the Wistar (Jla: Wistar) and Sprague Dawley (Slc:SD) strains were

used in this study. After the sacrifice by bloodletting under overdose anesthesia with ether, the samples were excised from the animals and examined for the immunohistochemistry and the immunocrossreactivities between antibodies and rat-antigens. For the histochemistry, samples were taken from the non-glandular and glandular (cardiac, fundic, and pyloric glands) stomach, duodenum, jejunum, ileum, cecum, proximal and distal colon, and rectum. For the immunocrossreactivities, samples were taken from the liver, fundic stomach and striated muscle.

Antibodies: CA-I and CA-II were purified from the equine erythrocytes [3], and CA-III from the equine striated muscle, respectively, as in the previous report [14]. Antisera to purified CA-I, CA-II and CA-III were raised against rabbits, respectively. One mg of each purified CA-I, CA-II or CA-III was dissolved in 0.5 ml PBS (pH 7.4), emulsified by addition of 0.5 ml Freund's complete adjuvant, and injected subcutaneously into the dorsal skin of rabbits. The immunization was followed by 5 injections of the same amount of the enzyme at intervals of a week. Rabbits were bled via the auricular vein at 10 days after the last injection. The antibodies to CA-I, CA-II and CA-III were separated from rabbit antisera by precipitation with 33% saturated ammonium sulphate solution. Each precipitate was dissolved in phosphate-buffered saline (PBS, 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) and dialyzed against this

buffer. The concentration of each immunoglobulin G (IgG) fraction was adjusted to 1.8 mg/ml. Normal rabbit IgG (1.8 mg/ml), prepared by the same method as described above, was used in place of antisera to CA isozymes for control examinations.

Determination of the immunocrossreactivities between antibodies and antigens: The crude samples extracted from the liver, fundic stomach and striated muscle in 0.01 M PBS, pH 7.4, with 1 mM 1, 4-dithiothreitol (Sigma, U.S.A.) and proteinase inhibitor cocktails (1 mM ethylenediaminetetraacetic acid disodium salt dihydrate, Sigma, U.S.A.; 1 mM p-chloromercuribenzenesulfonic acid, Sigma, U.S.A.; 1 mM pepstatine, Peptide Lab., Japan) were prepared for the determination of the immunocrossreactivities. After electrophoresis with 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), the gel was blotted on the immobilon transfer membranes (Millipore, U.S.A.) 4 times by Milliblot-SDA (Millipore, U.S.A.) [11]. The first by blotted membrane was stained for the protein bands with Coomassie Brilliant Blue R-250 (Sigma, U.S.A.). Other three membranes were immunostained with anti-CA-I, CA-II and CA-III antibodies, respectively, and visualized with 3, 3'-diamino-benzidine tetrahydrochloride (DAB) (Wako, Japan) in the presence of hydrogen peroxide [13].

Immunohistochemistry: Tissue pieces were fixed in Bouin's solution overnight, dehydrated in a graded series of ethanol, and embedded in the Paraplast (Monject, U.S.A.). Serial sections were made at 4 μ m, deparaffinized and rehydrated as usual, pretreated with 3% H₂O₂ in 99.5% methanol for 5 min at room temperature, and incubated with 2% normal goat serum in 0.01 M PBS, pH 7.4, for 20 min at 37°C. They were then incubated with anti-equine CA-I, CA-II and CA-III IgG, or normal rabbit IgG (for the negative control) for 1 hr at 37°C, and followed by the treatment with the avidin-biotin-peroxidase complex method (Vectastain Elite ABC-POD Reagent Kit, Vector, U.S.A.). Finally, each section was counterstained with hematoxylin and observed under a light microscope.

RESULTS

Immuno-blotting test: The anti-equine CA-I, CA-II and CA-III antibodies were confirmed to react with the rat isozymes, respectively, by the use of

immuno-blotting method. Single reactive band against each CA isozyme was indicated at about 30 kDa in each sample lane such as liver, glandular stomach and striated muscle. On the blotting membranes, CA-I was reacted with extract from the liver, CA-II was strongly reacted with those from the glandular stomach and liver, and CA-III intensely from the liver and striated muscle (Fig. 1).

Immunohistochemistry: The results are summarized in Table 1. In the stomach, surface epithelial cells and parietal cells of the glandular region showed a positive immunoreactivity to CA-II, but the non-glandular region showed a negative reaction. In the large intestine, each CA isozyme (CA-I, CA-II and CA-III) was localized in the upper portion of intestinal glands, but absent in the small intestine. The upper portion of intestinal glands in the cecum and proximal colon were intensely positive to CA-I and CA-II and moderately positive to CA-III (Figs. 2a, 2b, 2c). These activities were gradually decreased from distal colon to rectum (Fig. 2d). Absorptive columnar cells in the lower portion of the intestinal glands and goblet cells lacked the immunoreactivity to CA-I, CA-II and CA-III. There were no differences between the Slc:SD strain and the Jla:Wistar strain, and between the male and the female in the present immunohistochemical findings.

Fig. 1. Immunoblots with anti-carbonic anhydrase isozyme sera after 10% SDS-PAGE. Lanes 1, 4 and 7 represent the crude liver extract, lanes 2, 5 and 8 the crude stomach extract, lanes 3, 6 and 9 the crude striated muscle extract. Lanes 1, 2 and 3 are immunoreactive to anti-equine CA-I antibody, lanes 4, 5 and 6 to anti-equine CA-II antibody and lanes 7, 8 and 9 to anti-equine CA-III antibody, respectively. Reacted bands to anti-CA isozyme are located at about 30 kDa.

Fig. 2. Paraffin sections of rat cecum (a, b, and c) and rectum (d), showing immunohistochemical staining with anti-equine CA-I (a and d), anti-equine CA-II (b) and anti-equine CA-III (c) IgG as the primary antibody. Each immunoreactivity is noted in the absorptive columnar cells in the upper portion of the intestinal glands. No activity is noted in the goblet cells and the absorptive columnar cells in the lower portion of the intestinal glands. $\times 160$.

Table 1. Distribution of carbonic anhydrase isozymes in the rat gastrointestinal tract

Histological Sites	Isozymes		
	CA-I	CA-II	CA-III
Fundic Stomach			
Surface epithelial cells	— ^{a)}	+~++	—
Parietal cells	—	+~++	—
Cecum			
Columnar epithelial cells in the upper portion of glands	+++	+++	++
Proximal Colon			
Columnar epithelial cells in the upper portion of glands	+++	+++	++
Distal Colon			
Columnar epithelial cells in the upper portion of glands	++	+~++	±~+
Rectum			
Columnar epithelial cells in the upper portion of glands	+	+	±

a) Intensity of staining was graded subjectively on a scale of - representing nonreactivity to +++ representing very intense dark brown to black staining.

DISCUSSION

Crossreactivities of anti-equine CA-I, CA-II and CA-III to the rat antigens: Crossreactivities among antisera to rat CA-I, CA-II and CA-III were examined by radioimmunoassay to report the maximum crossreactivity of 0.01% [8]. Nishita and Matsushita [17] also examined the crossreactivity of anti-equine CA-III serum to CA-I and CA-II by enzyme immunoassay, and obtained the maximum crossreactivity of 0.035%. Anti-equine CA-III serum did not react with CA-I or CA-II in the erythrocytes of rats according to the immunohistochemical method [16]. In the present study, the single reactive band against CA-III was detected at about 30 kDa in the extracts from the liver and muscle in rats by the use of immunoblotting method. This finding coincides well with the previous report [15]. The single reactive band against CA-I or CA-II was indicated at about 30 kDa each sample lane such as liver and/or glandular stomach as in CA-III. Monospecific polyclonal antisera to equine CA isozymes are thus sufficiently selective for the immunohistochemical study in the rat.

Immunohistochemistry: Recently, Spicer *et al.* [20] examined the expressions of CA-II and CA-III in the large intestine of the mouse by immunohistochemical methods, and revealed that CA-III was strongly positive in the cecum and proximal colon and weakly positive in the distal and rectosigmoid colon; immunoreactivity to CA-II was strong and roughly similar throughout the mouse colon. In our study, however, each immunoreactivity to CA isozymes (CA-I, CA-II and CA-III) in the rat tended to decrease toward the distal digestive tract, such as from distal colon to rectum. The immunohistological distribution of CA-III in the rat intestinal tract was similar to that in the mouse [20], while the distribution of CA-II was different from that in the mouse [20]. The immunohistolocalization of CA-I has never been reported in the rodents. Thus, the distribution pattern of CA isozymes in the large intestine was different between rats and mice. These findings suggest that the functions of each segment of the rat large intestine may be different from those of the mouse. In addition, biochemical studies indicated that the proximal colon showed the significantly higher CA activity than the distal colon [2]. Although our data are not quantitative, they would appear to support these biochemical findings.

On the other hand, the present immunohisto-

chemical observations have agreed with previous histochemical investigations, as follows. The previous studies [10, 12, 18, 19] of CA with the improved Hansson's technique [6, 21] showed an intense cytoplasmic staining pattern in nongoblet superficial cells as well as cells lining the upper one-third to one half of the crypt as shown in the present study of CA-II with immunohistochemical technique. In the present study on the rat stomach, surface epithelial cells and parietal cells of the glandular region showed a positive immunoreactivity to CA-II. This pattern was consistent with that in the guinea pig [12], human [19], mouse [18], and rat [10]. These immunohistochemical observations agreed with the previous investigation [18] of CA-II in the mouse stomach. This may suggest that CA-II plays the same role in the rat and mouse stomach.

The expression patterns of CA isozymes in the large intestine suggested that the ion-transportations and acid-base condition of the lumen were supported by co-activations of each CA isozyme. It has been shown that the large intestine has the ability to secrete HCO_3^- into the lumen apparently in exchange for Cl^- absorption [4] and that the apical membrane from the rat colonocyte makes the $\text{Na}^+\text{-H}^+$ exchange [1, 5]. CA is related to these transport of acid and base by the epithelium. Recently, Suzuki and Kaneko [22] showed that CO_2 derived from the epithelial metabolism was hydrated by CA in the cell to make H^+ enter the mucosal solution while HCO_3^- enter the serosal solution, and suggested that the H^+ exit across the mucosal membrane was mediated by $\text{H}^+\text{-ATPase}$.

Taking these reports into consideration, the present results suggest that the differences of CA localizations in the large intestine may reflect different levels of the water absorption and the transport of acid and base.

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