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Characterization of metaplastic and heterotopic epithelia in the human gastrointestinal tract by the expression pattern of acyl-CoA synthetase 5

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Summary. Metaplastic and heterotopic epithelia are frequently found in the human intestine. The recently cloned human acyl-CoA synthetase 5 (ACS5) is a key enzyme in providing cytosolic acyl-CoA thioesters. The aim of the study was to identify and to locate the expression of ACS5 in the gastric body and the small intestine with metaplasia or heterotopia by different methods. In the normal gastrointestinal tract, ACS5 was predominantly found in the villus epithelium of the small intestine, but not in the gastric mucosa. Of note, strong expression of ACS5 was also detectable in intestinal metaplasia of the stomach. Inversely, ACS5 expression could neither be detected in heterotopic gastric mucosa of the corpus type nor in gastric, pseudopyloric, or antral metaplasia of the small intestine. In conclusion, our data implicate that ACS5 is a suitable differentiating marker molecule in the gastrointestinal tract.

Key words: ACS5, Gastrointestinal tract, Metaplasia, Villus epithelium

Introduction

Metaplasia is defined as a potentially reversible change from a fully differentiated tissue into another. The phenomenon is different from another type of change in tissue architecture called congenital heterotopia in which there is a developmental basis for the abnormally located differentiated tissue, e.g. occurrence of corpus type mucosa in the duodenum (Jass and Walsh, 2001). True metaplasia is found in different locations of the whole intestine, but preferentially located in the gastric body and in this location it is generally designated as intestinal metaplasia (Walker, 2003). The term intestinal metaplasia has been used to describe the occurence of metaplastic tissues e.g. in the stomach that shares some features of differentiation with the small or large bowel mucosa (Spechler, 2004). The inverse histomorphological pattern is occasionally seen in the bowel, where metaplastic tissues of the gastric type are found. This type of metaplastic epithelia is preferentially established in the small intestine and designated as gastric, pseudopyloric, or antral metaplasia. Pathological conditions of the small intestine frequently associated with occurrence of heterotopic or metaplastic epithelia are Meckel's diverticulum and Crohn's disease (CD). In CD, metaplasia is in discussion as a pathogenetic mechanism responsible for the disruption of the intestinal mucosal barrier which is generally assumed to be an important mechanism in the pathogenesis of inflammatory bowel disease (Fiocchi, 1998).

Although intestinal metaplasia of the stomach has some histomorphological characteristics in common with the intestinal mucosal phenotype, there are an increasing number of studies providing evidence that cellular differentiation in metaplastic tissues is different from that found in normal mucosa (Wong et al., 2000; Inada et al., 2001). Moreover, experimental evidence is given from mucin (histo-)chemistry showing that intestinal metaplasia of the stomach is a heterogeneous phenomenon which is reflected by several classifications and definitions (Tatematsu et al., 2003). Following the proposal of Tatematsu and coworkers, all of the subtypes of gastric and intestinal mixed type intestinal metaplasia and a subtype of the intestinal type without Paneth cells belong to the incomplete intestinal metaplasia category while the subtype of intestinal type with Paneth cells corresponds to the complete type of intestinal metaplasia (Tatematsu et al., 2003). It is widely accepted that the incomplete type of intestinal metaplasia of the stomach is a special risk factor in developing gastric cancer. Therefore, a reliable detection and definition of metaplastic epithelia in biopsies of the stomach by routine histopathological and immunohistochemical techniques is of high importance. When assessing

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metaplasia and cancer risk, some effort was made in order to establish molecular marker molecules (Jass and Walsh, 2001). One important family of such molecules are mucins, consisting of a core protein and a carbohydrate component. Experimental evidence is given that the overwhelming number of variations in mucin structure in the normal gastrointestinal tract occurs at the level of carbohydrate side-chain structures, whereas a modified expression of core mucin proteins is preferentially found in pathological conditions such as intestinal metaplasia of gastric mucosa (Jass and Walsh, 2001).

Here we present a new potential marker molecule - the enzyme acyl-CoA synthetase 5 (ACS5; NCBI database AB033920) - which could be of diagnostic relevance in characterization of metaplasia and heterotopia in the gastrointestinal tract. In the human intestine, ACS5 is preferentially found in the small intestine and shows increasing expression levels from the crypts of Lieberkühn towards the villi. ACS5 catalyzes the initial step required for oxidation, elongation, and desaturation of fatty acids that is needed for the synthesis of complex lipids and acylated proteins as well as for a variety of signals that regulate cellular metabolism and gene expression. Here we demonstrate that ACS5 is a suitable marker molecule for the characterization of metaplastic and heterotopic epithelia in the human gastrointestinal tract.

Materials and methods

Tissues

Formalin-fixed and paraffin-embedded tissues/ biopsies (total, n=40) of the squamocolumnar junction/ Z-line (n=6, Barrett's oesophagus; mean age, 67 years; range, 38 to 76 years), the gastric body (n=14, intestinal metaplasia; mean age, 70 years; range, 32 to 85 years), the duodenum (n=5, heterotopic gastric mucosa; mean age, 57 years; range, 40 to 69 years), the terminal ileum from 10 patients with CD (mean age, 38 years; range, 22 to 52 years) and resections of Meckel's diverticulum (5 patients; mean age, 42 years; range, 25 to 63 years) were investigated by in situ techniques (immunohisto-chemistry and mRNA in situ hybridization). The use of human tissues for pathological purposes was approved by each patient and ethical permit of Heidelberg University. All diagnoses were established by conventional clinico-pathological criteria. In CD, H&E stained sections from paraffin embedded tissues were used to define the inflammatory degree of all tissues according to Truelove and Richards (Truelove and Richards, 1956). For molecular analysis, i.e. Western blot analysis, RT-PCR, and cloning of an ACS5 fragment, surgical specimens of normal terminal ileum from 10 patients (controls) with sporadic colonic cancer (mean age, 58 years; range, 39 to 69 years) were used. Mucosal tissue layers were mechanically dissected, immediately cooled in liquid nitrogen, and stored at -80 °C until use.

Tissue preparation and Western blot

Mucosal samples were homogenized in TRI reagent (Sigma, Deisenhofen, Germany). Afterwards, protein and RNA were extracted following the procedure described by Chomcyznski (1993). For protein measurements, the BioRad assay (BioRad, München, Germany) was used. Protein preparations in Laemmli buffer were stored at -20 °C until use. Proteins were separated by SDS-PAGE (7.5 %) and transferred to a PVDF membrane (Millipore Corporation, Bedford, MA, USA) by semi dry blotting. Proteins were visualized by the ECL substrate (Amersham Pharmacia Biotech, Little Chalfont, England) following the manufacturer's recommendations. Negative controls included blots in which the primary antibody was omitted.

Reverse transcription

The SuperScript II amplification system for first-strand cDNA synthesis was used following the manufacturer's suggestions (Invitrogen/ Life Technologies, Karlsruhe, Germany). Briefly, 5 µg of DNase-digested total RNA was used for oligo (dT) primed first-strand cDNA synthesis. Reverse transcription was followed by a RNase H digestion step (20 min at 37 °C). In control experiments transcription of a commercially provided RNA was performed using the enzyme reverse transcriptase or distilled water, respectively.

PCR

For qualitative analysis of ACS5 transcripts in mucosal specimens, conventional PCR was performed using the following set of ACS5 (NM_016234) primer: 5'-TTT TTG TAC ACG GGG AGA GC-3', 5'-ACA GGC TGT CAA TTT GGG TC-3'. PCR's were running on a Gene Amp PCR System 9700 (PE Applied Biosystems, Foster City, USA). The 35 PCR cycles consisted of: 95 °C; 10 sec/ 68-58 °C; 15 sec/ 72 °C;10 sec. Amplicon purity and integrity was analyzed on 1.5% agarose ethidium bromide gels.

Cloning of an ACS5 fragment and mRNA in situ hybridization

For mRNA in situ hybridization, a 970 bp amplicon covering exon 21 and an untranslated region of human ACS5 was synthesized by PCR using a set of primer: 5'-CAA CAT TGA AAG CAA AGC GA-3' and 5'-AAT AAG CCT GTT GTG TGG GC-3'. The PCR-bluntII-topo vector was used for Topo TA cloning (Invitrogen/ Life Technologies). Orientation of inserts was determined by end-sequencing. Purified plasmids were restricted with BamHI and then transcribed with T7 RNA polymerase (Roche-Diagnostics, Mannheim,

Germany) to generate antisense and sense RNA transcripts using DIG-labeled UTP. The transcripts were shortened by alkaline hydrolysis. mRNA in situ hybridization was performed on deparaffinized tissue sections treated with proteinase K (Roche-Diagnostics; 10 µg/ml; 30 min at 37 °C) and acetic anhydride. Hybridization with a probe concentration of 4 ng/µl or 10 ng/µl hybridization mixture was carried out (46 °C; overnight). After stringent post-hybridization washings, sections were then incubated with an AP-coupled anti-DIG antibody (Roche-Diagnostics). Specific signals were visualized using NBT and X-phosphate (Roche-Diagnostics) as chromogens. In control experiments, the antisense probe or the anti-digoxigenin antibody were replaced by the sense probe or blocking medium (Roche-Diagnostics), respectively.

DNA sequencing

Sequencing was performed using 300 ng plasmid DNA and 10 pmol of the appropriate set of primer. All reactions were run on an ABI 3700 capillary sequencer according to routine protocols suggested by the manufacturer (ABI, Weiterstadt, Germany).

Antibodies

In addition to mAb KD7 (Gassler et al., 2004), secondary HRP-conjugated anti-rat IgG antibodies were used (1:10,000; Santa Cruz, Santa Cruz, USA). The KD7 supernatant was used for both procedures, immunohistochemistry and Western blot analysis.

Immunohistochemistry

For immunohistochemistry, sections of paraffin-embedded tissues were used following routine protocols. Incubation with the primary antibody was performed for 1 h at room temperature in a moist chamber. Sections were then washed three times with PBS. The ABC detection kit with DAB as the chromogen was used in accordance with the manufacturer's suggested protocols (DAKO, Glostrup, Denmark). Negative controls included similarly processed sections where the appropriate normal serum was used or where the primary antibody had been totally omitted.

Results

Distinct expression of ACS5 in the villus epithelium of normal small intestine

In order to corroborate and to extend our initial experiments concerning mAb KD7 which recognizes ACS5 (Gassler et al., 2004), expression of ACS5 was investigated in the small intestinal mucosa by molecular techniques, e.g. conventional RT-PCR and Western blot analysis as well as in situ techniques, e.g. immunohistochemistry and mRNA in situ hybridization. As shown by these methods, ACS5 was abundantly expressed and synthesized in normal human small intestinal mucosa. Moreover, it could be shown that ACS5 expression was located in the villus epithelium by mRNA in situ hybridization and immunohistochemistry. A continuous cytoplasmic immunostaining of the villus epithelium including goblet cells was regularly found (Fig. 1A). ACS5 was not detectable in the normal gastric mucosa by the in situ techniques used.

Intestinal metaplasia of gastric mucosa

In the present study, intestinal metaplasia of the squamocolumnar junction/Z-line (Barrett's oesophagus) and the gastric body were analyzed by in situ techniques working in formalin-fixed and paraffin-embedded tissues. In Barrett's oesophagus, subtle metaplastic changes in the mucosa are sometimes difficult to recognize by conventional H&E light microscopy. However, these changes were demonstrable by ACS5 immunhistochemistry (Fig. 1B,C). Notably, strong expression of ACS5 protein was found in intestinal metaplasia of the gastric body (Fig. 1D). In order to corrobrate the findings by immunohistochemistry, mRNA in situ hybridization for ACS5 was performed on serial tissue sections. The ACS5 mRNA was found in an identical distribution pattern to the ACS5 protein (Fig. 1E,F).

Metaplasia of small intestinal mucosa

Metaplasia of the small intestinal mucosa describes the occurrence of mucoid differentiated cells which have some features in common with normal gastric mucosa. The phenomenon is termed gastric, pseudopyloric, or antral metaplasia and frequently found in CD.

In non-inflamed or inactively inflamed CD with metaplasia of the antral type, the continuous cytoplasmic ACS5 immunostaining of the villus epithelium was disrupted. This change was due to the presence of metaplastic epithelium in which ACS5 expression was not detectable (Fig. 1G). Thus, the lack of ACS5 expression is apparently helpful to identify subtle changes in the mucosal architecture of the small intestine, e.g. the appearance of a metaplastic immunophenotype.

In contrast, heterotopic gastric mucosa, which is found in about 30 % of Meckel's diverticulum (Oguzkurt et al., 2001), regularly intermingles with epithelial cells of the normal small intestine thus composing a hybrid mucosa. In order to visualize the cellular composition, immunostainings against ACS5 were performed demonstrating a loss of ACS5 in the heterotopic gastric mucosa when compared with the adjacent normal small intestinal mucosa (Fig. 1H).

Heterotopic gastric mucosa of the corpus type is frequently seen in the duodenum. The histological feature strongly resembles the hybrid mucosa in Meckel's diverticulum. Immunohistochemically, loss of

ACS5 was associated with the occurrence of the heterotopic mucosa (Fig. 1I). In all cases investigated, there was a clear discrimination of heterotopic tissues from normal surface epithelium (i.e. resident surface epithelium of the duodenum).

Discussion

The acyl-CoA synthetase 5 (ACS5) is involved in fatty acid metabolism by catalyzation of acyl-CoA molecules which are necessary for the synthesis of

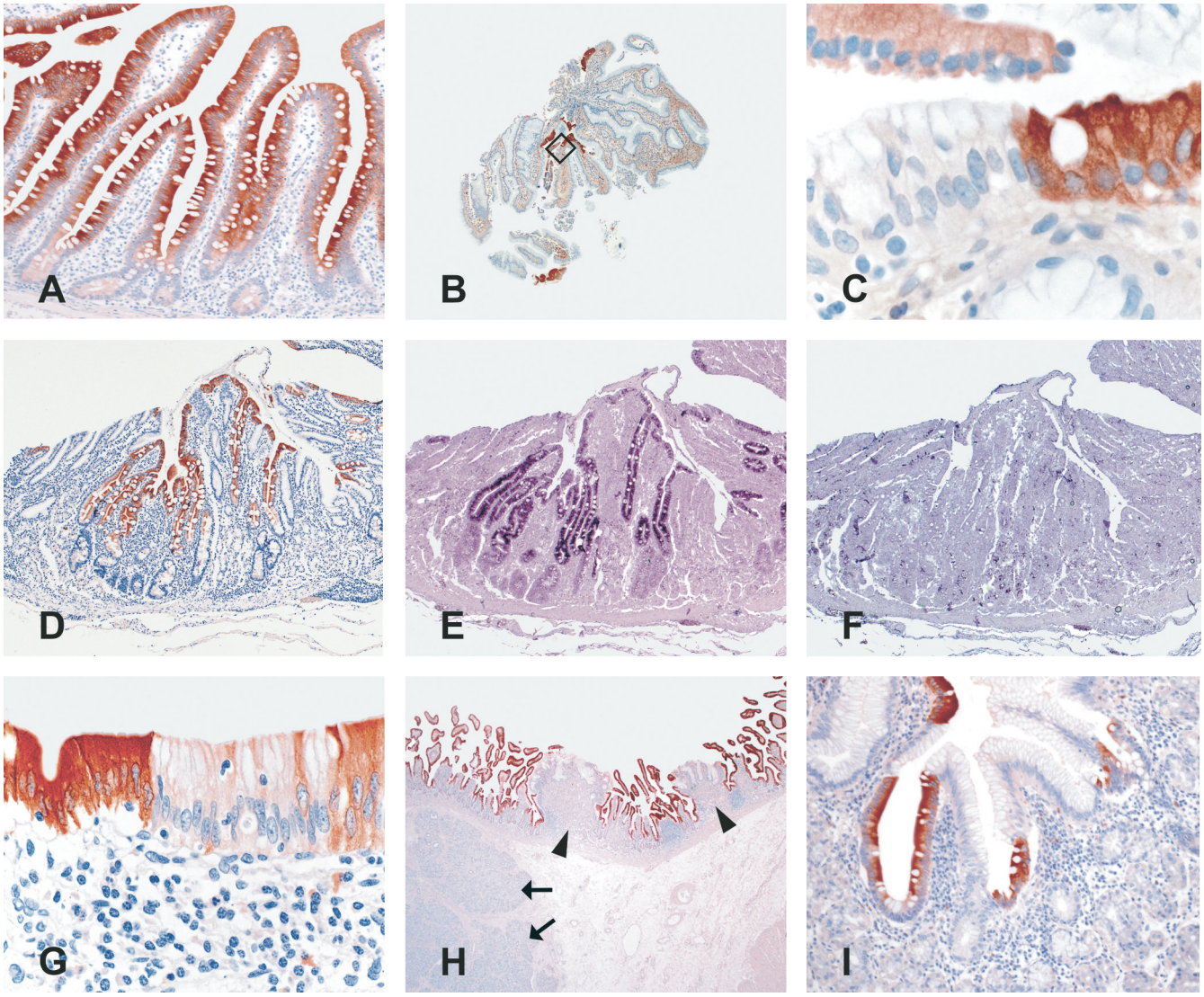

Fig. 1. Expression of ACS5 in normal small intestinal mucosa **(A)** and metaplastic epithelium in the squamocolumnar junction/Z-line **(B, C)**, in the gastric body **(D-F)**, and in the small intestine with Crohn's disease **(G)** or Meckel's diverticulum **(H)**, and in heterotopic gastric mucosa of the small intestine **(I)** by DAB-immunohistochemistry **(A-D, G-I)** and mRNA in situ hybridization **(E, F)**. **A.** In normal small intestine, ACS5 expression is found as a continuous cytoplasmic immunostaining of the villus epithelium including goblet cells (DAB-immunohistochemistry). **B.** Biopsy of Barrett's oesophagus after DAB-immunostaining against ACS5. Subtle changes in the mucosal architecture represent intestinal metaplasia expressing ACS5. The rectangle indicates the higher magnification shown in **C**. **C.** Biopsy of Barrett's oesophagus with highlighted location shown in **B** (rectangle). ACS5 is found in goblet cells and in columnar cells of the metaplastic epithelium, but not in normal cardiac epithelia. **D.** Synthesis of ACS5 is found in intestinal metaplasia within the stomach, whereas the gastric epithelium lacks ACS5 expression (DAB-immunohistochemistry). **E.** Serial tissue section from **D** after mRNA in situ hybridization using digoxigenin-labeled antisense riboprobes to ACS5. A strong signal is found in the metaplastic epithelium corroborating the results found by immunohistochemistry **D**. **F.** Serial tissue section from **D** and **E** after mRNA in situ hybridization using digoxigenin-labeled sense riboprobes to ACS5. **G.** Non actively inflamed CD of the small intestine. ACS5 immunostaining is not found in a subtle location in the vicinity of normally ACS5-expressing villus epithelium indicating for a metaplastic immunophenotype. **H.** Meckel's diverticulum with heterotopic gastric mucosa (arrowheads) and heterotopic pancreatic tissue (arrows). Loss of ACS5 in the surface epithelium is associated with the occurrence of heterotopic tissues. **I.** Duodenal biopsy with heterotopic gastric mucosa of the corpus type. ACS5 immunostaining is exclusively found in remnants of the original, autochthonal surface epithelium of the small intestine. Original magnification: A, x 25; B, H, x 5; C, G, x 63; D-F, x 10; I, x 40

complex lipids and acylated proteins as well as for signaling in cellular metabolism and gene expression (Black et al., 2000; Yamashita et al., 2000). In humans, the small intestine is one important location of fatty acid metabolism because of the uptake of alimentary lipids. The aim of this study was to perform an in-depth analysis of ACS5 expression and synthesis in the human gastrointestinal tract showing metaplasia of the surface epithelium.

In normal human gastrointestinal tract, different structural and functional specializations of the surface epithelium are found. Our experiments clearly show that ACS5, which is a key-enzyme in fatty acid metabolism, is preferentially located in the small intestine. Data from Western blot analysis and PCR technique were substantiated by immunohistochemistry showing an increasing ACS5 expression from the crypts of Lieberkühn to the villi in normal human small intestine, which is in accordance with data obtained in the rat (Oikawa et al., 1998). The spatial distribution of ACS5 in epithelial cells of the small intestinal mucosa implicates that ACS5 is useful as a new marker molecule for the crypt-villus-axis and epithelial homeostasis in the intestine as suggested by our group previously (Gassler et al., 2004). In order to evaluate epithelial homeostasis in the intestine, expression of mucins and other molecules, like enterocytin and activin, was extensively studied (Crow and Ong, 1985; Sonoyama et al., 2000; Jass and Walsh, 2001; Parnis et al., 2004). To our knowledge, there is no systematical study correlating the expression of molecules involved in fatty acid metabolism to pathophysiological conditions like metaplasia in the intestine.

The term metaplasia is used to describe the potentially reversible phenomenon that fully differentiated tissues may change into other types of differentiation (Wong et al., 2000; Walker, 2003; Spechler, 2004). Changing milieu conditions are of importance in pathogenesis and establishment of metaplasia. Here we show that strong expression of ACS5 is found in epithelial cells of intestinal metaplasia, but not in the normal gastric surface epithelium. Of note, gastric, pseudopyloric, or antral metaplasia as well as heterotopic gastric mucosa within the small intestine displayed the inverse pattern. In conclusion, the in situ analysis of ACS5 expression is a sensitive and specific tool for the detection and definition of metaplasia in the gastric body and in the small intestine. Our results give also indirect evidence that the enzymatic activies in fatty acid metabolism differ between the metaplastic/heterotopic and the original, autochthonal epithelium.

Unequivocal and sufficient detection and definition of metaplastic epithelia especially in small mucosal biopsies is of importance in diagnosis of Barrett's oesophagus and Crohn's disease. Our data give evidence that ACS5 immunohistochemistry could be very useful to explore metaplasia in such cases.

In summary, ACS5 is preferentially found in the small intestinal mucosa with highest amounts being expressed in the surface epithelium of villi. Importantly, strong expression of ACS5 could be found in intestinal metaplasia of the gastric body, but not in gastric, pseudopyloric, or antral metaplasia of the small intestine. Moreover, ACS5 expression could not be found in heterotopic gastric mucosa. The results show that ACS5 is a suitable marker molecule in the precise detection and definition of metaplasia and heterotopia in the gastrointestinal tract.

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