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ORIGINAL PAPER

**Siru Haila · Ulpu Saarialho-Kere
Marja-Liisa Karjalainen-Lindsberg · Hannes Lohi
Kristiina Airola · Christer Holmberg
Johanna Hästbacka · Juha Kere · Pia Höglund**

The congenital chloride diarrhea gene is expressed in seminal vesicle, sweat gland, inflammatory colon epithelium, and in some dysplastic colon cells

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Abstract Congenital chloride diarrhea (CLD) is an autosomal recessive disorder of intestinal electrolyte transportation caused by mutations in the anion transporter protein encoded by the down-regulated in adenoma (DRA), or CLD, gene. In this study, in situ hybridization and immunohistochemistry were performed to investigate the expression of CLD in extraintestinal normal epithelia and in intestinal inflammatory and neoplastic epithelia. The expression of the closely related anion transporter diastrophic dysplasia sulfate transporter, DTDST, was also examined and compared with that of CLD in colon. The only extraintestinal tissues showing CLD expression were eccrine sweat glands and seminal vesicles. In inflammatory bowel disease and ischemic colitis, expression of CLD mRNA in colon epithelium was similar to histologically normal colon epithelium, but the protein was found deeper in crypts, including proliferative epithelial cells. In intestinal tumors, the expression pattern of CLD was dependent on the differentiation status of the tissue studied: epithelial polyps with no or minor dysplasia showed abundant expression, whereas adenocarcinomas were negative. The DTDST gene was abundantly expressed in the upper crypt epithelium of colonic mucosa.

S. Haila (✉) · H. Lohi · J. Hästbacka · J. Kere · P. Höglund
Department of Medical Genetics, Haartman Institute,
P.O.Box 21 (Haartmaninkatu 3), 00014 University of Helsinki, Finland
e-mail: Siru.Haila@helsinki.fi
Tel.: +358-9-1911, Fax: +358-9-19126677

U. Saarialho-Kere · K. Airola
Department of Dermatology,
00029 Helsinki University Central Hospital, Finland

M.-L. Karjalainen-Lindsberg
Department of Pathology, Haartman Institute,
00014 University of Helsinki, Finland

C. Holmberg
Hospital for Children and Adolescents,
00029 Helsinki University Central Hospital, Finland

Introduction

Congenital chloride diarrhea (CLD) is an autosomal recessive disorder of intestinal electrolyte transportation (Norio et al. 1971). A defect in ileal and colonic absorption of Cl^- in exchange for HCO_3^- results in a lifelong acidic diarrhea with a high chloride content (Holmberg et al. 1977; Holmberg 1986). CLD is caused by mutations in the down-regulated in adenoma (DRA) gene (Höglund et al. 1996) which was originally cloned as a candidate tumor suppressor gene due to its downregulation in colon adenomas and adenocarcinomas (Schweinfest et al. 1993). The CLD protein is highly homologous to two other known human members of the sulfate transporter gene family, namely the human diastrophic dysplasia gene (DTDST) and the human Pendred syndrome gene (Hästbacka et al. 1994; Everett et al. 1997). Mutations in the human DTDST gene cause disorders of skeletal development such as diastrophic dysplasia (Hästbacka et al. 1994), atelosteogenesis type II (Hästbacka et al. 1996), and achondrogenesis type IB (Superti-Furga et al. 1996). By Northern analysis it is expressed in a wide variety of human tissues, including colon (Hästbacka et al. 1994), whereas studies on rat dtdst have suggested a more restricted expression profile limited mainly to cartilage and intestine (Satoh et al. 1998).

CLD mRNA has been shown to be expressed in the mature surface epithelium, especially in brush border cells of normal ileum and colon (Byeon et al. 1996; Höglund et al. 1996) whereas no expression studies on DTDST have been published. These proteins share common features such as suggested transmembrane topology and function as anion transporters. The CLD protein has been shown to act as a Na^+-independent Cl^-/OH^- or Cl^-/HCO_3^- exchanger (Melvin et al. 1999; Moseley et al. 1999), but it transports also other anions such as sulfate and oxalate (Silberg et al. 1995). The DTDST protein transports sulfate (Hästbacka et al. 1994; Satoh et al. 1998) which has a major role in the pathogenesis of cartilage growth defects.

In this study, we have characterized epithelial tissues and specific cell types that express CLD *in vivo*, which is an important step in elucidating physiological functions of the protein and pathophysiology of chloride diarrhea as a disease entity. The effect of inflammation on CLD expression was examined in specimens of several types of colitis. Since the CLD gene has been suggested to play a role in neoplastic transformation or tumor progression in colon (Schweinfest et al. 1993; Antalis et al. 1998), a set of normal tissues, benign neoplasms, and adenomas with dysplasia, as well as invasive carcinomas, were studied to obtain a better understanding of this process. Since by Northern analysis, the rat dtdst has been shown to be expressed mainly in cartilage and intestine, human DTDST was included in this study to elucidate its possible expression and distribution in human colon.

Materials and methods

Tissues

Formalin-fixed, paraffin-embedded archival specimens from adult patients were obtained from the Department of Pathology, Haartman Institute, University of Helsinki. The following specimens were examined: Crohn's (colon; *n*=5), ulcerative (colon; *n*=6), and ischemic colitis (colon; *n*=4); hyperplastic polyps (*n*=5), villous (*n*=3) and tubular (*n*=3) adenomas, colonic adenocarcinomas (*n*=5); mastopathy (*n*=5), lactating mammary gland (*n*=2), endometrium at different menstrual phases (*n*=10), dermatofibroma (*n*=1), scalp skin with alopecia (*n*=1), and prostate hyperplasia (*n*=1). Samples from normal ventricle (*n*=4), duodenum (*n*=5), jejunum (*n*=3), ileum (*n*=6), appendix (*n*=2), colon (*n*=4), sigma (*n*=1), rectum (*n*=1), anal canal (*n*=2), liver (*n*=4), pancreas (*n*=3), parotid gland (*n*=3), skin (*n*=4), heart (*n*=1), lung (*n*=3), kidney (*n*=3), prostate (*n*=9), seminal vesicle (*n*=3), and placenta (*n*=4) were studied as well.

In situ hybridization

A 622-bp fragment corresponding to positions 1650–2271 of the published human CLD/DRA cDNA (GenBank number: L02785) was generated by polymerase chain reaction (PCR). This fragment was designed with a T7 RNA polymerase promoter at the 3′ end and an SP6 RNA polymerase promoter at the 5′ end. Both sense and antisense probes were transcribed from this PCR product using the riboprobe in vitro transcription system (Promega, Madison, Wis., USA). Antisense and sense RNA probes were labeled with alpha-^{35}S-UTP and purified probes were used at 4×10^5 cpm/µl of hybridization solution. The DTDST probe was constructed in a similar way corresponding to the nucleotide positions 1440–2061 of the DTDST sequence (GenBank number: U14528), corresponding to the homology region of the CLD probe.

As previously described (Prosser et al. 1989; Höglund et al. 1996), deparaffinized 5-µm tissue sections were digested with 0.6–1.0 µg/ml proteinase K for 30 min at 37°C and treated with 0.25% acetic anhydride in 0.1 M triethanolamine buffer for 10 min at room temperature. Hybridization was carried out overnight at 52°C. After hybridization, the slides were washed under stringent conditions, including RNase A, and exposed to LM-1 emulsion (Amersham, Aylesbury, UK) for 21–60 days at 4°C. The slides were developed and counterstained with hematoxylin and eosin. Normal colon samples known to be positive were used as controls in each experiment and a sense RNA probe was used as a negative control (Höglund et al. 1996).

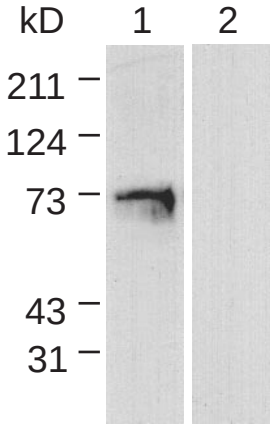

Fig. 1 Western blot detection of the congenital chloride diarrhea (CLD) protein. The band of approximately 75 kDa is specifically recognized by anti-CLD serum (*1*), while preimmune serum of the same rabbit detects no specific bands from homogenized colon epithelium (*2*). Molecular size markers are indicated in kilodaltons

Immunohistochemistry

Antiserum was raised in rabbits against the synthetic peptide FNPSQEKDGKIDFT corresponding to amino acids 2375–2416 of the published cDNA sequence (GenBank number: L02785). Peptide synthesis and antibody production were purchased from Research Genetics (Huntsville, Ala., USA). The specificity of the antibody was demonstrated by Western blotting (Fig. 1) using homogenized human colon epithelium. After centrifugation at 12000 *g* for 10 min, the supernatant was collected and diluted 1:2 in Laemmli sample buffer (Pharmacia, Uppsala, Sweden) containing 5% β-mercaptoethanol. Denatured proteins were separated on a 4–20% polyacrylamide gradient gel and the gel was blotted onto Hybond C-extra (Amersham) membrane using standard protocols. The primary antibody serum was diluted 1:500. Biotin conjugated anti-rabbit IgG (Boehringer Mannheim, Mannheim, Germany) 1:2000 in 0.1% Tween 20/PBS containing 5% non-fat milk was used as the secondary antibody and was then detected using streptavidin-POD (Boehringer Mannheim, Mannheim, Germany) 1:5000 in 0.1% Tween 20/PBS containing 5% non-fat milk. The protein bands were visualized by chemiluminescence according to standard protocols.

Serial sections to those used for in situ hybridization were used for immunohistochemistry. The peroxidase–antiperoxidase technique was performed using an automatic immunostaining device (Ventana Medical Systems, Tucson, Ariz., USA) and Ventana kits. For pretreatment, the deparaffinized slides were boiled in a microwave oven for 15 min in 10 mM citrate buffer (pH 7.0). Anti-CLD serum was diluted 1:100–1:200 and diaminobenzidine was used as the chromogenic substrate. Preimmune serum was used as a negative control in parallel sections.

To assess proliferative activity of epithelial cells, Ki-67 staining was performed. For pretreatment, deparaffinized sections were boiled in 10 mM citrate buffer for 3×5 min. After primary antibody reaction with rabbit anti-human Ki-67 antigen (Dako, Glostrup, Denmark), the slides were processed with StreptABComplex/HRP Duet kit (Dako) utilizing the biotinylated secondary antibody followed by a complex of streptavidin and biotinylated peroxidase. Aminoethylcarbazole was used as a chromogenic substrate and slides were counterstained with Mayer's hematoxylin.

Results

Extraintestinal normal tissues

Our previous Northern analysis revealed strong expression of CLD mRNA in colon and prostate (Höglund et al. 1996). In this study both stromal and epithelial tissues of the prostate itself were constantly negative for

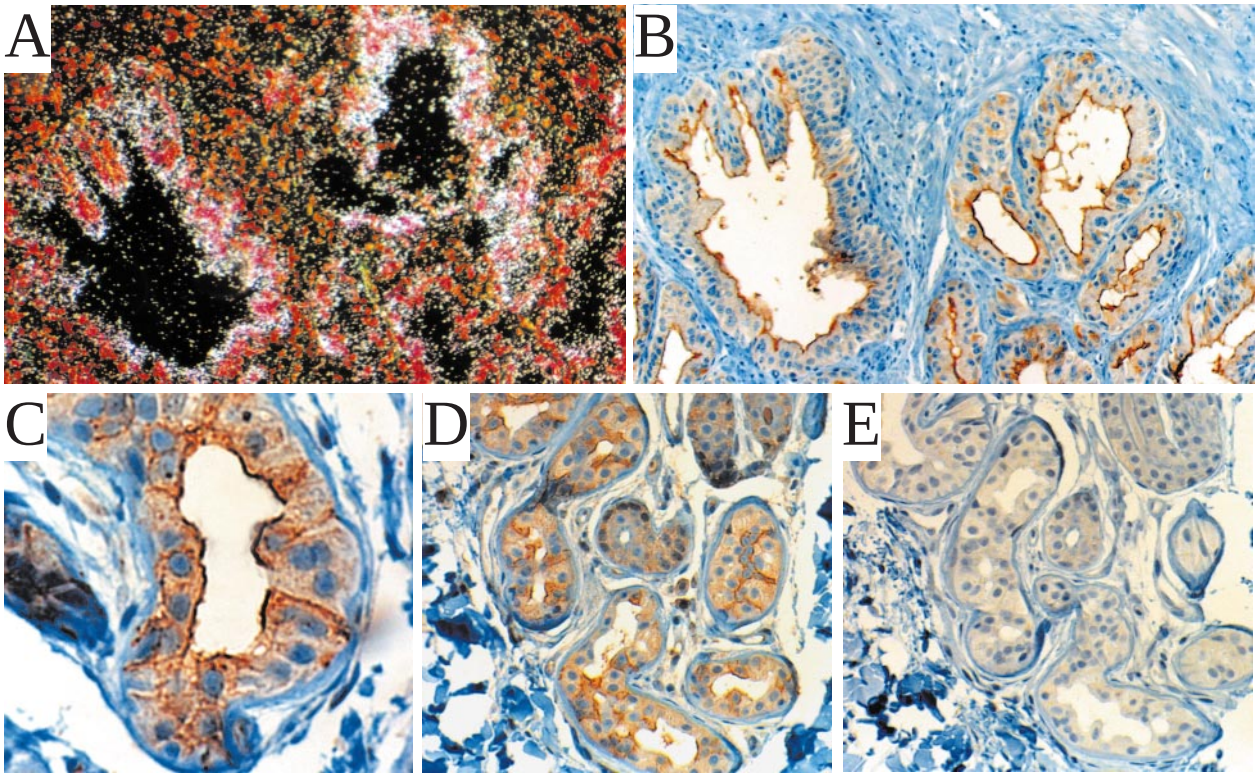

Fig. 2A–E CLD is expressed in seminal vesicles and eccrine sweat glands. In seminal vesicle, strong signal for CLD mRNA (**A**; darkfield image) and protein (**B**) are seen in luminal border of ductal epithelium. Similarly, the luminal side of secretory portion of the eccrine sweat gland demonstrates intensive CLD immunoreactivity (**C,D**) while no signal is detected in sections processed with preimmune rabbit serum (**E**). Original magnification: **A,B** ×100; **C** ×400; **D,E** ×200

CLD mRNA and protein, while signal was found in intra- and extraprostatic seminal vesicles (Fig. 2A,B). Strong immunoreactivity was observed especially on microvilli of the secretory columnar cells of the ductal epithelium (Fig. 2B).

Among other epithelial tissues studied only eccrine sweat glands showed CLD protein expression. Abundant immunoreactivity was detected on the luminal side and in intercellular canaliculi of epithelial cells in the coiled secretory part of the eccrine sweat gland (Fig. 2C–E). Ventricle, duodenum, liver, pancreas, parotid gland, small salivary glands, lung, heart muscle, prostate, endometrium, placenta, mammary gland, and kidney were repetitively negative for both CLD mRNA and protein.

Normal colon and hyperplastic polyps

We have previously shown that in normal colon CLD mRNA is detected in the surface epithelium, mainly in the absorptive epithelial and goblet cells, whereas the signal was absent at the bottom of the crypts (Höglund et al. 1996). By immunohistochemistry, normal colon epithelium showed intense CLD expression both at the apical side of nucleus in the cytoplasm (Fig. 3A–C) and focally in the brush border (Fig. 3C) of the epithelial cells.

Hyperplastic polyps, generally considered to lack malignant potential, demonstrated cytoplasmic expression of the CLD protein identical with the normal epithelium (Fig. 3D), but no brush border staining could be detected due to a total lack of brush border in this set of samples. Hyperplastic polyps showed similar distribution of proliferating epithelial cells by Ki-67 immunostaining when compared to normal colonic mucosa (data not shown).

Inflammatory bowel tissues

To assess possible alterations in CLD expression in response to inflammation, altogether 15 samples of ulcerative, Crohn's, and ischemic colitis were studied by in situ hybridization and immunohistochemistry. No significant change in the expression of CLD mRNA and cytoplasmic immunoreactivity for the CLD protein was detected in the colon mucosal surface in any samples studied. Even cases with active inflammation (Fig. 3E–G) and the gut epithelium bordering ulcerations (Fig. 3H) demonstrated normal cytoplasmic immunoreactivity. In Crohn's and ischemic colitis also the brush border staining was similar to normal control (Fig. 3I), but was missing in samples with ulcerative colitis.

In serial sections of inflammatory intestinal samples, Ki-67 immunostaining showed constant redistribution of the proliferative cell compartment to the upper crypt re-

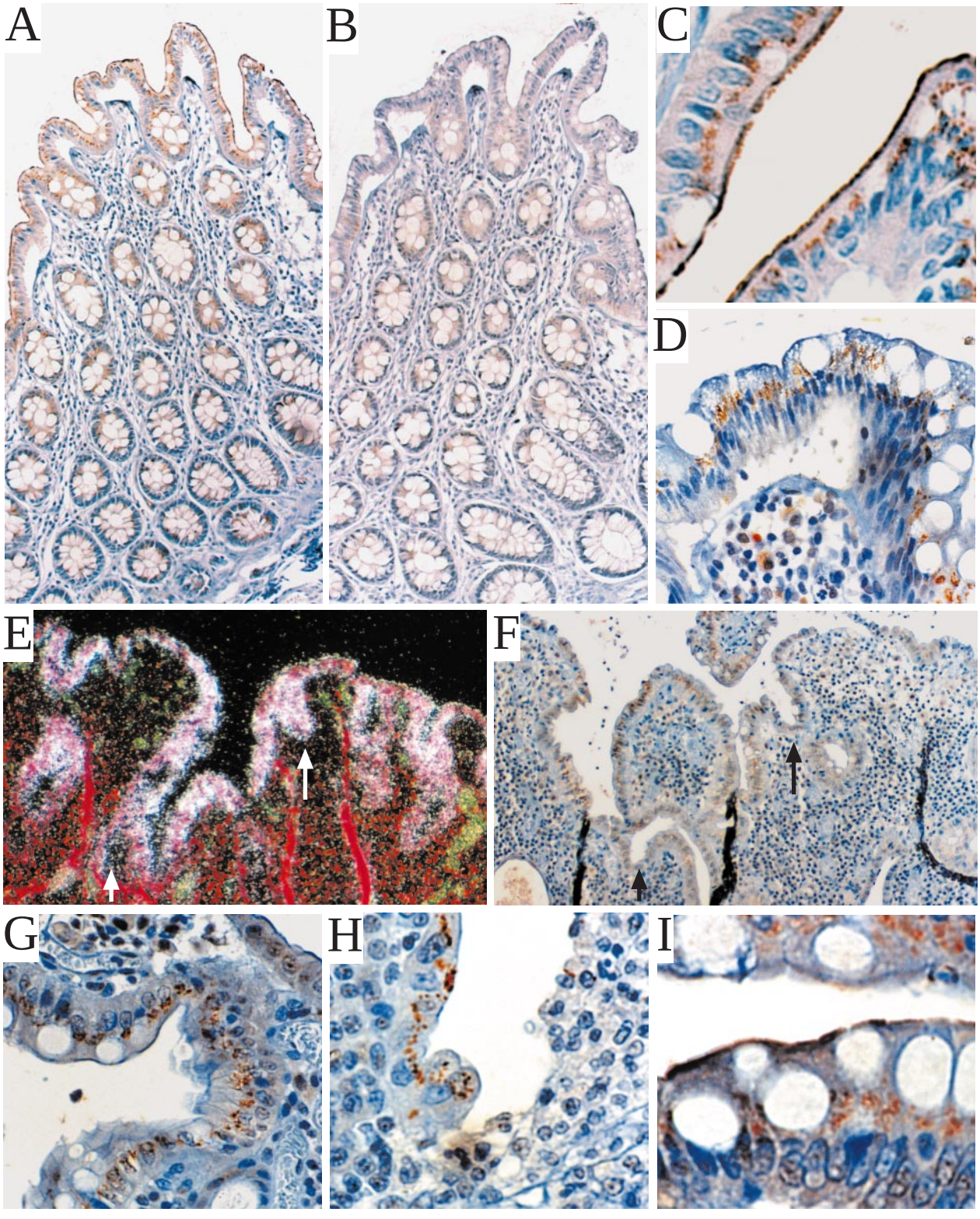

Fig. 3A–I Expression of the CLD protein in normal, hyperplastic, and inflammatory colon. **A,C** Immunostaining for CLD protein is detected in the apical surface of absorptive enterocytes in histologically normal colon tissue. **B** No immunoreactivity is seen on sections processed with preimmune rabbit serum. **D** Hyperplastic polyps showed cytoplasmic expression of the CLD protein similar to the normal epithelium. **E,F** Strong signal for CLD mRNA (**E**) as well as immunoreactivity for CLD protein (**F**) are detected in a severely inflamed ulcerative colitis specimen in nearby sections. *Arrows* mark corresponding structures. **G** Higher magnification of the same specimen. **H** Expression is present even in epithelium bordering ulcerations. **I** Brush border staining could also be detected focally, here shown in Crohn's colitis. Original magnification: **A,B,E,F** ×100; **C,H** ×400; **D,G** ×200; **I** ×500

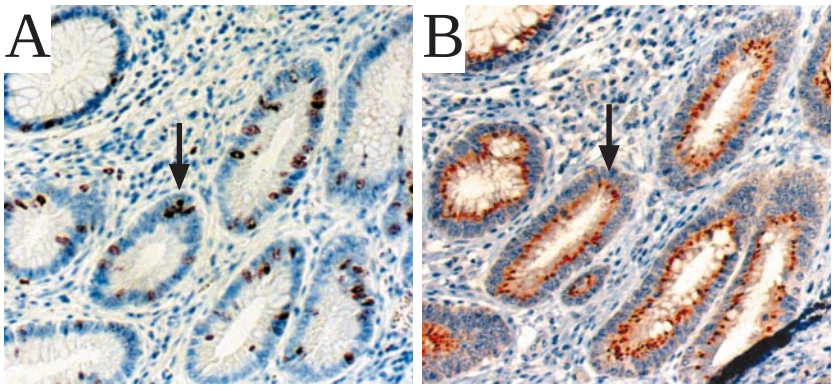

Fig. 4A,B In inflammatory bowel disease the expression of CLD is found also in the areas with proliferative activity. Colocalization of Ki-67 (**A**) and CLD (**B**) in the lower crypt regions in nearby sections of Crohn's colitis specimens. *Arrows* mark corresponding structures. Original magnification: **A,B** ×100

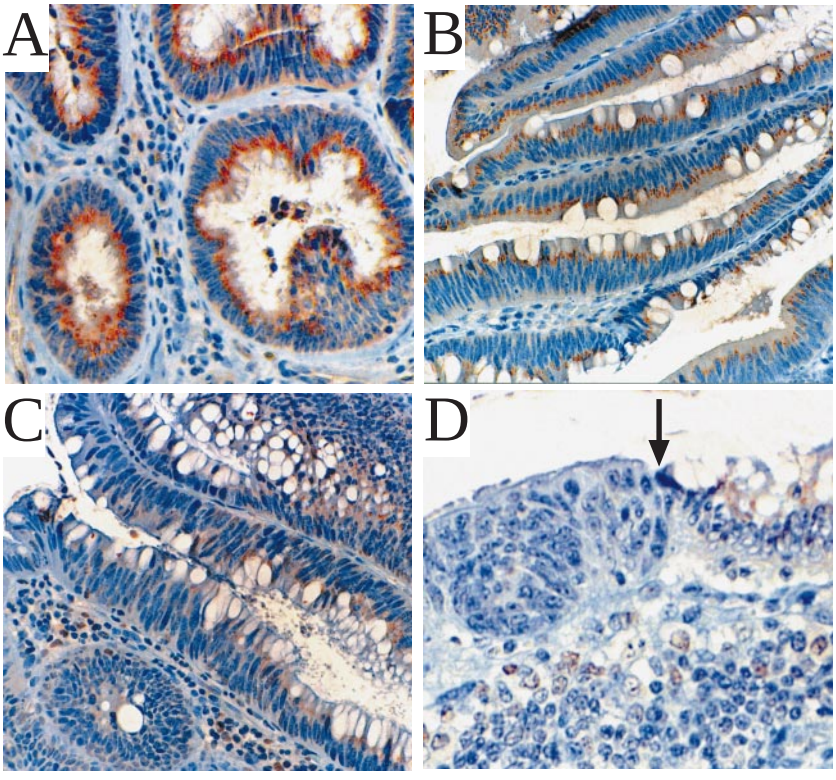

Fig. 5A–D CLD is expressed in some dysplastic colon cells. **A–C** Tubular (**A**) and villous (**B**) adenomas with minor dysplastic features demonstrated only slightly decreased apical cytoplasmic immunoreactivity, while in **C** the expression is more clearly downregulated in adenomas with moderate dysplasia. **D** No CLD-specific immunoreactivity is detected in an adenocarcinoma. *Arrow* marks the border line between adenocarcinoma and normal epithelium. Original magnification: **A–C** ×100; **D** ×200

gions. Colocalization of the Ki-67 and CLD signal in the lower crypt regions suggested an ability of proliferating cells to express CLD (Fig. 4A,B).

Proliferative epithelial lesions of the colorectum

Multiple colorectal proliferative lesions and carcinomas were studied to elucidate whether CLD contributes to cellular mechanisms leading to neoplastic transformation or tumor progression. Proliferative lesions of the colorectum revealed expression of CLD that was associated with the state of differentiation of the tumor: highly differentiated but proliferative lesions showed expression similar to normal epithelium, whereas poorly differentiated invasive tumors lacked expression. Both tubular and villous adenomas with minor dysplastic features showed slightly reduced cytoplasmic immunoreactivity (Fig. 5A,B), but with moderate dysplasia the cytoplasmic immunoreactivity was clearly reduced (Fig. 5C). Total lack of expression was observed in adenomas with severe dysplasia and in adenocarcinomas (Fig. 5D).

Diastrophic dysplasia sulfate transporter

Intense colonic expression of the closely related human DTDST gene was detected in the epithelial cells of the upper one-third of the normal crypts (Fig. 6A,B,D). At this region the CLD signal was normally present (Fig. 6C). Expression of DTDST decreased through the opening of the crypt and was absent both in the luminal surface epithelium and in the bases of the crypts (Fig. 6B).

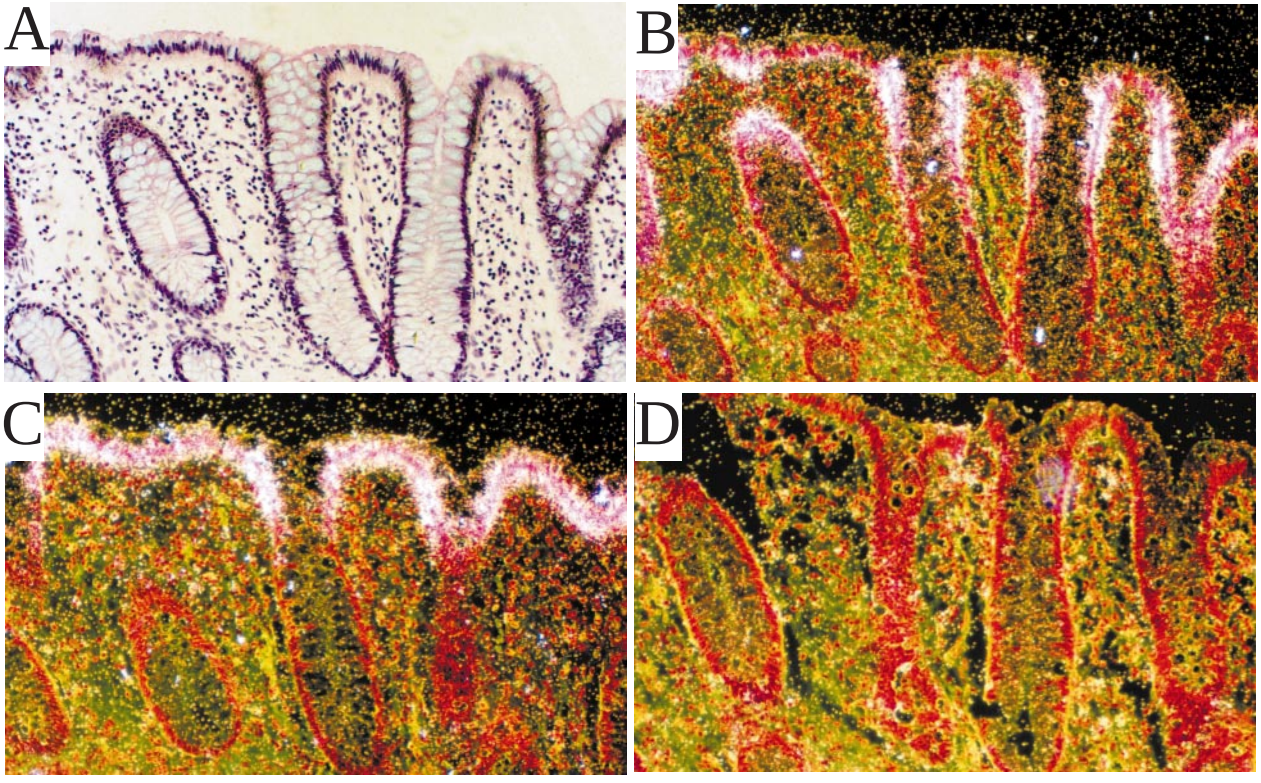

Fig. 6A–D The diastrophic dysplasia sulfate transporter (DTDST) gene is abundantly expressed in colonic upper crypt epithelium. **A** Brightfield view of a section hybridized with DTDST antisense probe. **B** In a darkfield image of the same section, DTDST mRNA is expressed in the epithelial cells of the upper one-third of the normal crypts where in darkfield image of parallel section (**C**) CLD expression was present. **D** No signal was detected in darkfield view of a serial section with the DTDST sense negative control probe. Original magnification: **A–D** ×100

Discussion

We have investigated the pattern of expression of the human CLD gene in various normal glandular tissues, as well as in histologically normal, inflammatory, hyperplastic, and neoplastic intestinal tissues using in situ hybridization and immunohistochemistry. We also studied the colonic expression pattern of the closely related anion transporter, DTDST. In normal colon, most abundant CLD mRNA and protein expression were demonstrated in the mature colon surface epithelium, especially at the apical side of the nucleus in the cytoplasm and in the brush border of absorptive enterocytes. Brush border staining corresponds well with the mature, functional form of the protein at the membrane. In contrast, the role and function of the abundant cytoplasmic protein remains to be clarified. Most likely it reflects a newly synthesized form of the CLD protein which is under terminal processing, or retarded form in an intracellular compartment, such as endoplasmic reticulum or Golgi apparatus, and finally targeted to the plasma membrane.

Interestingly, CLD expression was found in the seminal vesicle epithelium and not in prostate as our earlier Northern analysis suggested. It seems now obvious that the prostate sample in our commercial Northern filter also contained intraprostatic seminal vesicular tissue that might be difficult to separate from glandular posterior part of prostate. The seminal vesicle fluid contains a high concentration of HCO_3^- (Okamura et al. 1985), which is suggested to be secreted by an active epithelial transport system (Mann and Lutwak-Mann 1981). CLD might well be involved in this process since its intestinal manifestation includes a defect in Cl^-/HCO_3^- exchange (Holmberg et al. 1975). At ejaculation, stored sperm mix with the alkalic seminal vesicle fluid and the motility of the primarily quiescent sperm is rapidly activated. Defects in the neutralization process result in the acidity of the luminal fluid, causing hypomotility of the sperm and infertility/subfertility (Breton et al. 1996). This hypothesis is supported by the finding that low levels of bicarbonate have been associated with hypomotile spermatozoa (Okamura et al. 1986). It is important to re-evaluate the clinical significance of the seminal vesicular expression of the CLD gene in male CLD patients.

Considering the complexity of sweat secretion physiology, the finding of CLD protein expression in the secretory sweat gland epithelium is not surprising even though no abnormalities concerning sweat secretion are known in CLD patients. The mechanisms of electrolyte transportation across cellular borders and to the sweat gland lumen are not well established but complicated cooperation of multiple electrolyte transporters has been proposed. The main role has been suggested to belong to

Na-K-2Cl cotransporters (Sato et al. 1989) that have been recently shown to be present and regulated in simian eccrine clear cells (Toyomoto et al. 1997). At least during cholinergic sweat secretion Cl^{-}-HCO_3^{-} and Na-H exchangers have also been implicated in the control of sweat composition (Sato et al. 1989). Localization of the CLD protein to the coiled secretory part of the eccrine sweat gland suggests it to be one of the mediators of electrolyte transportation involved in primary sweat formation.

In inflammatory bowel disease (IBD), the disturbed balance between proinflammatory and anti-inflammatory cytokines has been suggested to play an important role in maintaining chronic inflammation (Casini-Raggi et al. 1995; Noguchi et al. 1998). Cytokines have also been shown to alter epithelial ion transport and permeability (McKay and Baird 1999), although little is known about the regulation of enterocytic protein expression by inflammatory mediators. Previously, inflammation and IL-1 stimulation have been reported to reduce the expression of the CLD gene (Yang et al. 1998). Here we show that colonic mucosa with IBD, compared with normal colonic surface mucosa, show no significant change in the expression of the CLD gene or cytoplasmic form of the CLD protein. In contrast, the brush border immunostaining was present in samples with Crohn's and ischemic colitis, but could not be detected in ulcerative colitis samples. These findings suggest that reduced CLD expression reported earlier is not a constant phenomenon in inflammatory intestinal tissue.

Increased proliferative activity in IBD has already been previously shown in terms of expansion of the proliferative zone of the colonic crypt up to the lower two-thirds of the crypt (Serafini et al. 1981). In our set of samples with IBD CLD is expressed also in these areas suggesting that the expression of CLD is not limited to non-proliferating cells, although in normal tissues, no CLD expression is usually observed in the cells of the proliferative cell compartment.

Originally, a role of CLD/DRA in colon carcinogenesis was proposed due to its downregulation in adenomas and adenocarcinomas (Schweinfest et al. 1993). Further studies have suggested the downregulation to correlate with tumor progression and to be particularly significant in the earliest phases of neoplastic evolution (Antalis et al. 1998). Little clinical support to this hypothesis was given by an epidemiological survey studying incidence of cancer among CLD patients with a non-functional V317del germline mutation and their parents (obligatory carriers). The total cancer incidence was unchanged, although a slightly elevated risk of colon cancer was found (Hemminki et al. 1998). However, since patients homozygous for this mutation thrive well without prominent cancer predisposition, a major role of CLD in the initiation of cancer is unlikely. These findings led us to study systematically the expression of CLD in a set of different colonic tumors, including all stages of colon tumor progression, to clarify whether the downregulation of the CLD gene is associated with neoplastic transformation and progression.

In adenomatous polyps with mild to moderate dysplasia, the cytoplasmic signal was reduced but clearly present in all samples. However, there were no regions with brush border immunoreactivity normally found in the apical membrane. In more advanced dysplasias and adenocarcinomas also the cytoplasmic staining was gradually lost, whereas the non-neoplastic epithelium adjacent to tumors expressed CLD normally. We propose that there is a gradual loss of expression that is most likely associated with the decreased differentiation status of those tissues. Our results show that CLD is intensely expressed in normal and benign colon surface epithelium and that its downregulation is a common phenomenon, but a later event in neoplastic evolution of colonic mucosa than previously thought. The significance of this finding remains open; however the proposed tumor-suppressor capability of CLD appears unlikely. More likely, loss of expression reflects loss of terminal differentiation in colon surface epithelium.

The human DTDST gene was strongly expressed in colon with distribution to the top one-third of the crypt and lacking signal at the surface level. This localization was partially overlapping with that of CLD. Clinical abnormalities known in diastrophic dysplasia are restricted to cartilage and bone and no intestinal manifestations have been reported. In vitro functional studies have demonstrated that both CLD and DTDST mediate the transport of sulfate (Silberg et al. 1995; Satoh et al. 1998) that is inhibited by extracellular chloride. In physiological circumstances, CLD transports chloride preferentially (Moseley et al. 1999), whereas the loss of sulfate transport in cartilage has been shown to be the major source of cartilage pathology in diastrophic dysplasia. The physiological role of DTDST in human colon and regulation of its expression require further studies. However, the clinical phenotype of CLD suggests that DTDST is unlikely to be able to compensate CLD transport function in human intestine.

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