

Autoimmunity Against Human Tropomyosin Isoforms in Ulcerative Colitis

Localization of Specific Human Tropomyosin Isoforms in the Intestine and Extraintestinal Organs

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Background: Tropomyosins (TMs) are microfilament cytoskeletal proteins, and 5 major human TM isoforms (hTM1–5) are described. hTMs, particularly isoform 5 (hTM5), is capable of inducing autoantibodies and T-cell response in ulcerative colitis (UC). However, cellular localization of hTM isoforms in the colon and in extraintestinal organs commonly involved in UC is unknown.

Methods: Using isoform-specific monoclonal antibodies, we localized hTMs through immunoperoxidase assay in normal colon ($n = 12$), small intestine ($n = 14$), esophagus ($n = 10$), skin ($n = 19$), eye ($n = 12$), gallbladder ($n = 16$), liver, including bile duct at the porta hepatis ($n = 4$), lungs ($n = 4$), and pancreas ($n = 4$).

Results: There is intense expression of hTM5, but not other isoforms, in the epithelium of the colon, gallbladder, and skin. In the eye, hTM5 is expressed only in the nonpigmented ciliary epithelium. Although extrahepatic and interlobar large ductal biliary epithelium was positive, bile canaliculi at the portal tract were negative. The immunoreactivity in epithelial cells from these organs is diffuse cytoplasmic and along the periphery. In colon epithelium, there is intense expression along basolateral areas and luminal (apical) surface. In the small intestinal epithelium, however, hTM5 expression is weak and distinctly different than in the colon. hTM5 was not detected in the squamous epithelium of the esophagus, although it was strongly positive in the skin. hTM1, hTM2, and hTM3 are localized predominantly in smooth muscle of the intestine and blood vessel wall but not in the epithelium. hTM4 is localized in the endothelial cells and basement membrane of the colonic epithelium.

Conclusions: hTM5 is the predominant isoform in the epithelium of colon and extraintestinal organs commonly involved in UC. The

unique expression of hTM5 may allow its interaction with effector immune cells involved in the immunopathogenesis of UC and its extraintestinal manifestations.

Key Words: tropomyosin, autoimmunity, ulcerative colitis, extraintestinal manifestations

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Tropomyosin (TM) isoforms are cytoskeletal microfilamental proteins that are involved in the stabilization of actin filaments, intracellular organelle movement, cell shape maintenance, and cytokinesis.¹ Eight human TM (hTMs) isoforms have been detected in human fibroblasts, with high and low molecular weights ranging from Mr 30K to 40K.² Organ-specific isoform(s) and multiple isoforms in the same cell have been demonstrated.¹ Autoimmunity has been implicated in the pathogenesis of ulcerative colitis (UC). Recent studies, both in patients with UC^{3,4} and in experimental animal models of colitis,^{5,6} demonstrated that TMs or TM-related peptides⁷ may be a putative target autoantigen in UC. Autoantibodies in UC were found to be mainly against hTM5, which is the predominant hTM5 isoform in colon epithelium.⁸ The preferential occurrence of serum antibodies against an unknown colonic antigen(s) in UC was first reported in 1959,⁹ and they are found in the sera and produced locally by colonic lymphocytes in 75% of UC patients.¹⁰ We reported that the autoantibody produced locally by plasma cells in the colonic mucosa is directed against hTM5 in >90% of UC patients.¹¹ Colonic mucosa-bound IgG antibody in the colon of UC patients was called colitis-colon-associated IgG antibody.¹² This antibody was originally reported to bind with a 40-kDa colonic protein (p40).¹³ The p40 antigen was purified, partially sequenced, and found to be 93% identical to TMs.³

TMs are capable of inducing significant autoimmune response.¹⁴ In a computer-based physicochemical and biochemical analysis of 109 human autoantigens, several sequences of TM residues were among the most potent autoantigens.¹⁴ Anti-TM antibodies have been demonstrated in an animal

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model of colitis developed in T-cell receptor (TCR) knockout (TCR $\alpha^{-/-}$) mice.^{5,6} In this model of TCR $\alpha^{-/-}$ mice, the titer of anti-TM antibody correlated with the histological severity of colitis. About two thirds to three fourths of sera from patients with UC reacts with purified TMs.^{3,4} Increased IgG1 antibody directed against colonic extract enriched in TMs is produced by lamina propria lymphocytes from patients with symptomatic UC. Such a local immune response against TMs is not seen in the lamina propria cells from normal colon and in the colonic mucosa of patients with symptomatic Crohn's disease.¹⁵ More recently, we reported specific T-cell response against hTM5 in UC but not in Crohn's disease.¹⁶

In one of the largest population-based studies from a county in Stockholm, Sweden, with a population of 1.5 million individuals, 1279 patients had UC, of whom 271 patients (21%) had extracolonic manifestations restricted mainly to the joints, skin, eyes, and biliary tract.¹⁷ A strong positive correlation was demonstrated between extracolonic manifestations and extent of colonic involvement and familial UC. Familial aggregation of UC in association with extracolonic diseases supports the concept of autoimmunity involving UC and extracolonic organs. Hepatobiliary complications had the highest incidence, occurring in 11% of patients with UC.¹⁷ hTM5 also may be an autoimmune target for extracolonic organs; indeed, autoantibody against a specific TM peptide was reported in 100% of patients with primary sclerosing cholangitis.⁷

Little is known about the cellular localization of hTM isoforms in the intestinal and extraintestinal organs commonly involved in UC. In the present study, using a sensitive immunoperoxidase assay, we have examined the cellular localization of hTM isoforms in the normal colon, small intestine, and extraintestinal organs commonly involved in UC.

MATERIALS AND METHODS

Antibodies

The isoform-specific monoclonal antibodies (mAb) against 5 isoforms of hTM have previously been described^{1,18-23}

TABLE 1. Monoclonal Antibodies Against hTMs and Their Immunoglobulin Isotypes

Monoclonal Antibody	Immunoglobulin Isotype	Recombinant hTM to Which the Ab Reacts	References
CG1	IgG1	hTM1	18
CG β 6*	IgM	hTM2/hTM3	18, 19
LC24	IgG1	hTM4	1, 21
CG3	IgM	hTM5	18-20

*CG β 6 reacts with both hTM2 and hTM3.

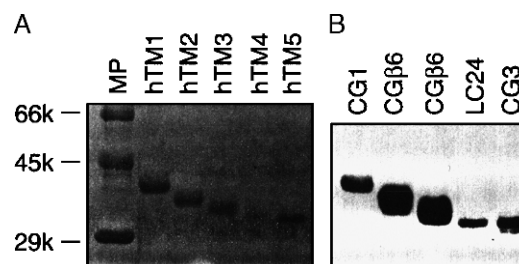


FIGURE 1. Immunotransblot analysis with the mAbs against various hTM isoforms (hTM1-5). A, Recombinantly purified hTM isoforms analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Coomassie brilliant blue staining. B, Each of the 5 isoforms (hTM1-5) was probed with the isoform-specific monoclonal antibodies: CG1 for hTM1, CG β 6 for hTM2/3, LC24 for hTM4, and CG3 for hTM5. A clear reactivity against each of the respective isoforms is evident. MP indicates marker proteins.

(Table 1). The monospecificity of these mAbs has been confirmed against recombinant hTM isoforms⁸ by ELISA, immunoprecipitation, and Western blot analysis. The CG1 mAb (IgG1 subtype) reacts specifically with hTM1. The mAb CG β 6 (IgM isotype) reacts with both hTM2 and hTM3. At present, we do not have an mAb to differentiate between hTM2 and hTM3. LC24 (IgG1 subtype) reacts specifically to hTM4, and CG3 mAb (IgM isotype) reacts with the hTM5 isoform.¹⁸⁻²⁰ Unrelated mouse mAbs of IgM and IgG1 isotypes (MOPC) were used as controls (Sigma Chemicals, St Louis, Mo).

Human Tissues

A total of 95 formalin-fixed, paraffin-embedded archival tissue specimens were used for immunohistochemical staining experiments. The samples consisted of the following: colon ($n = 12$), small intestine ($n = 14$), esophagus ($n = 10$), skin ($n = 19$), eye ($n = 12$), gallbladder ($n = 16$), liver, including bile duct at the porta hepatis ($n = 4$), lungs ($n = 4$), and pancreas ($n = 4$). All tissue specimens were examined by routine histological examination and were found to be normal, except for specimens of the gallbladder, liver, lungs, and pancreas.

Immunohistochemical Staining

The assay was performed using a modification of a method previously described.^{24,25} Briefly, 5- μ m-thick sections from formalin-fixed paraffin-embedded surgical or biopsy specimens were deparaffinized and rehydrated in decreasing concentrations of ethyl alcohol. Free aldehydes were reduced with phosphate-buffered saline (pH 7.4) containing 0.05% sodium borohydrate for 20 minutes at 4°C. Sections were then successively incubated with monoclonal anti-tropomyosin antibodies and biotinylated rabbit anti-mouse IgM or IgG (Dako, Carpinteria, Calif) for

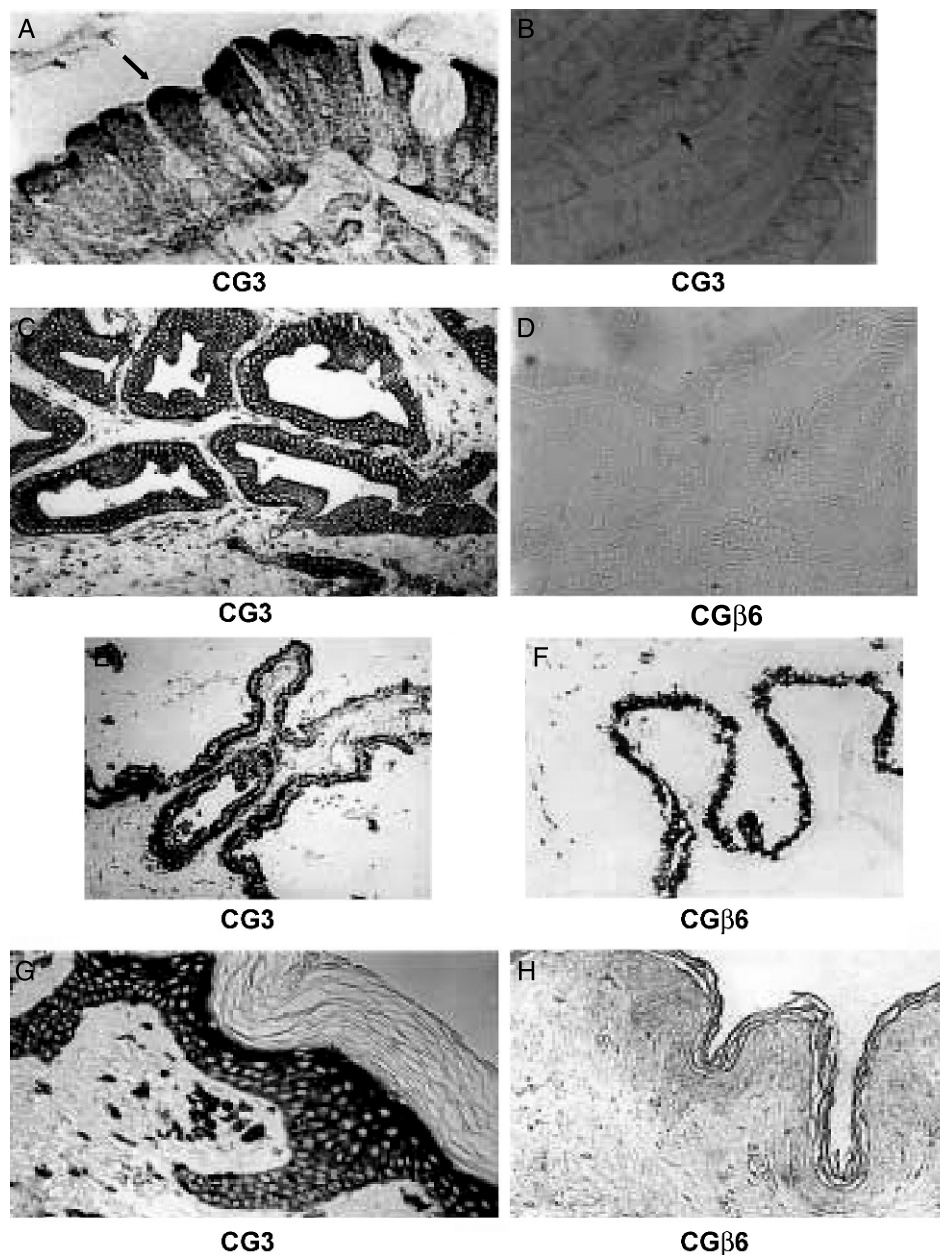
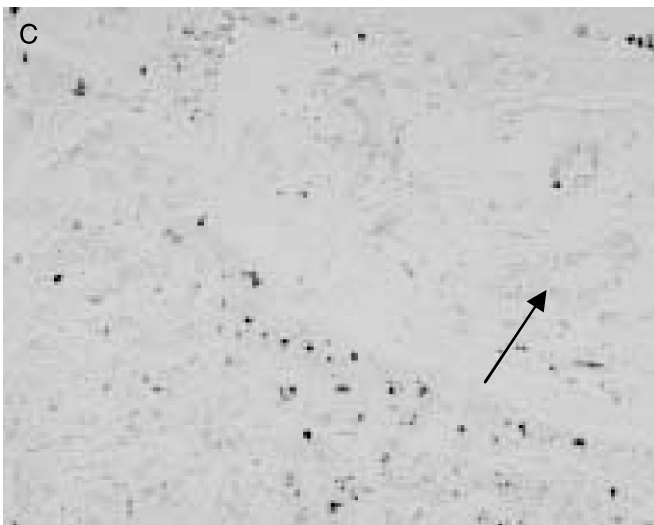
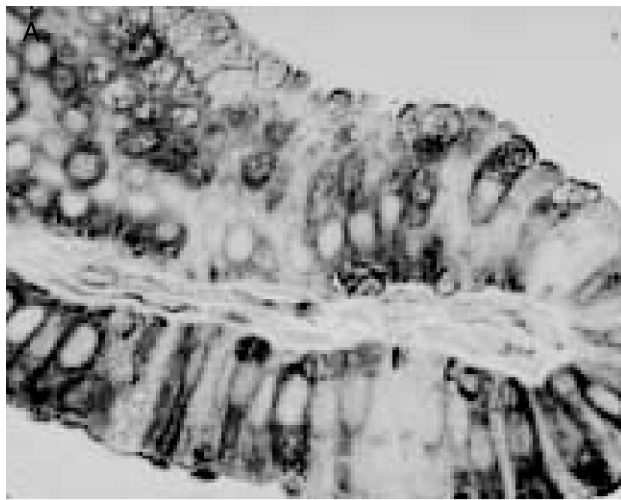


FIGURE 2. hTM5 expression in normal colon (A), ileum (B), gallbladder (C), ciliary body in the eye (E), and skin (G). hTM5 expression in the colonic mucosa is strongly cytoplasmic, and a dense bandlike reactivity is evident along the brush border area (arrow) and basal areas. The reactivity in the ileum is faint cytoplasmic, and no reactivity is evident along the brush border (arrow). Strong reactivity with CG3 monoclonal antibody (anti-hTM5) is evident in the biliary epithelium from a gallbladder mucosa, nonpigmented ciliary epithelium (outer layer) in the eye, and keratinocytes in the skin. CGβ6 (anti-hTM2/3) reacted with neither the epithelium of the colon and ileum (not shown) nor gallbladder (D), eye (F), and skin (H). Pigmented layer (inner layer) of ciliary process is seen in both E and F.

30 minutes each at 37°C. Parallel sections were incubated with appropriate unrelated mouse mAbs of IgM or IgG1 isotypes. Hydrogen peroxide (3%) for 20 minutes at room temperature, streptavidin-peroxidase (Dako) for 30 minutes at 37°C, and 3-3' diaminobenzidine (10 mg/mL in Tris HCl buffer, pH 7.6, containing 0.03% hydrogen peroxide) for

20 minutes at room temperature were successively added. Slides were then dehydrated, mounted, and observed under light microscopy. In between each incubation, extensive washings in phosphate-buffered saline were performed. The presence of clear brown staining was graded as positive; its absence was graded as negative or zero. The intensity of



positive staining was assessed on a scale of 1+ to 3+: 1+ = definite but mild reactivity, 2+ = moderate reactivity, and 3+ = strong reactivity. To examine the specificity of the positive staining, several tissue sections from the colon and small intestine were examined with the mAbs after preabsorption of the antibodies with respective recombinant hTMs. The presence of reactivity without preabsorption and its absence following absorption with the specific hTMs indicated specificity of the staining.

RESULTS

Table 1 summarizes the mAbs against various hTM isoforms, including the references in which they are reported in detail. Figure 1 shows the isoform specificity of the anti-hTM mAbs examined by transblot analysis against recombinantly synthesized hTM isoforms. There was no cross-reactivity of the mAbs against various other hTM isoforms, except that the mAb CG β 6 reacts with both hTM2 and hTM3.

In the immunoperoxidase experiments, the epithelium of colon, biliary epithelium of the gallbladder, extrahepatic bile duct, intrahepatic large bile ducts (interlobar), and squamous epithelium of the skin predominantly expressed the hTM5 isoform (Fig. 2). However, nonepithelial tissue such as connective tissue, blood vessels, and smooth muscle did not express hTM5. Other hTM isoforms such as hTM1–3 were not detected in the epithelial cells. There were distinct differences in terms of intensity and cellular localization of hTM5 between colonic and small intestinal epithelial cells. CG3 (anti-hTM5) mAb strongly reacted with colonic epithelial cells, and the immunoreactivity was most intense along the brush border areas and basolateral domain of colonocytes (Fig. 2A). There was also diffuse cytoplasmic staining. In contrast, the staining pattern of CG3 mAb in the small intestine was much weaker, and the localization was distinctly different (Fig. 2B). In small intestinal enterocytes, the immunoreactivity was faint and exclusively cytoplasmic, and in contrast to colonocytes, there was no reactivity in the brush border areas of small intestinal enterocytes (Fig. 2B). CG3 did not react with squamous epithelium of the esophagus. However, it reacted intensely with the squamous epithelium of the skin (Fig. 2G).

The immunoreactivity of CG3 in the biliary epithelial cells is strong, diffuse cytoplasmic and more dense along the periphery of the cells (Fig. 2C and Fig. 3, A and B). This reactivity is intense in the epithelium of gallbladder (Fig. 2C), extrahepatic bile ducts (Fig. 3A), and large bile

FIGURE 3. hTM5 expression (CG3) in the extrahepatic biliary epithelium (A) and an intrahepatic large bile duct (interlobar) epithelium (B). C, However, the bile canaliculi (arrow) in the portal triad did not react with CG3. Also note that hepatocytes and periductal tissue did not react with CG3 (A–C).

ducts (interlobar) (Fig. 3B). However, no reactivity is detected in the bile canaliculi at the portal tracts (Fig. 3C). In the eye, hTM5 was expressed only in the nonpigmented ciliary epithelium (Fig. 2E); the rest of the eyeball tissue was totally negative for hTM5. CG3 did not react with lungs (Fig. 4) and pancreatic tissue, except for some staining of islet cells (data not shown).

In contrast to hTM5 expression, the mAb against hTM1, CG1, reacted with neither epithelial cells of the large or small intestine nor with biliary epithelial cells (Fig. 5A) and skin (Fig. 5B). However, it reacted strongly along the basement membrane of colonic crypts, blood vessel wall, and muscularis mucosa (Fig. 6). The mAb against hTM2/3, CG β 6, also did not react with the epithelial cells from the gastrointestinal tract, including both large and small intestine, gall bladder epithelium (Fig. 2D), eye (Fig. 2F), and

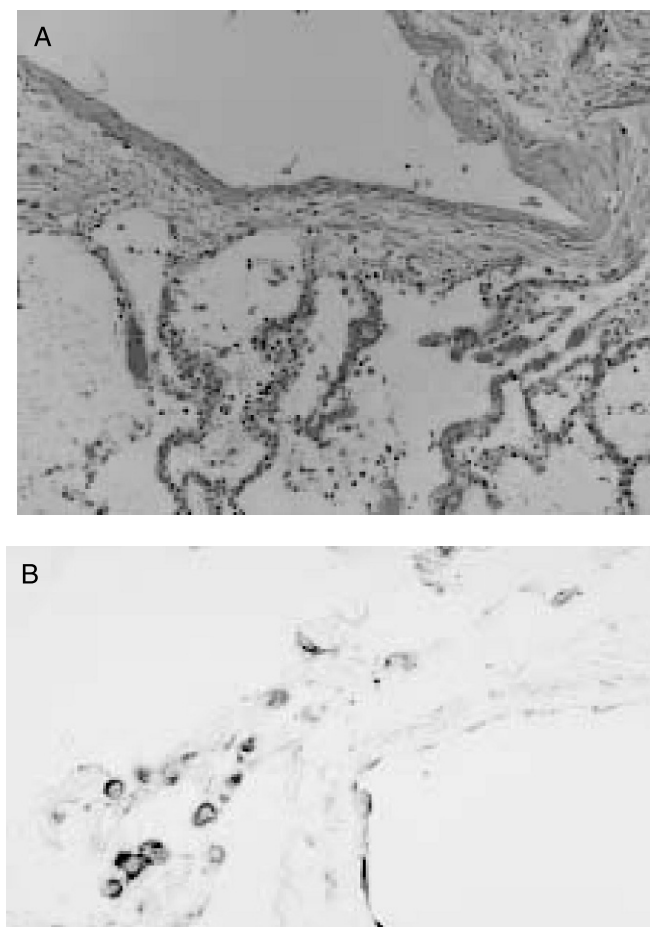


FIGURE 4. A tissue section from lung stained with hematoxylin and eosin (H&E; A) and the serial section stained with CG3 (B). There was no reactivity with alveolar epithelium. The reactivity seen in some of the cells is due to intrinsic peroxidase activity in macrophages and neutrophils.

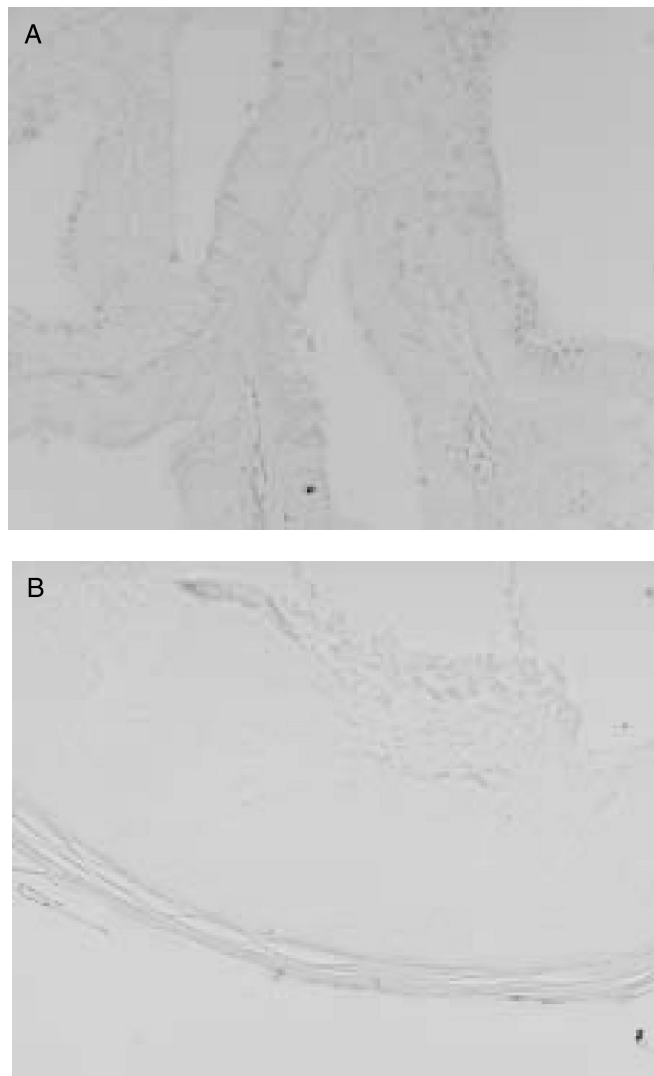


FIGURE 5. Nonreactivity of CG1 (anti-hTM1) against gallbladder epithelium (A) and squamous epithelium in the skin (B).

squamous epithelium of the skin (Fig. 2H). However, it reacted with smooth muscles (not shown).

In colonic epithelium, the LC24 mAb (anti-hTM4) had minimal reactivity in the cytoplasm. However, strong staining was noted with mononuclear cells in lamina propria (Fig. 7). There was no reactivity along the apical (brush border) areas of the colonocytes. Mild cytoplasmic staining also was noted in the small intestinal enterocytes.

DISCUSSION

Using the sensitive immunoperoxidase assays and hTM isoform-specific mAbs, we have shown that there is a selective distribution of known hTM isoforms (hTM1–5) in the intestinal and extraintestinal organs commonly affected in UC. Intestinal epithelium, but not esophageal epithelium, contains predominantly hTM5 and a small amount of hTM4,

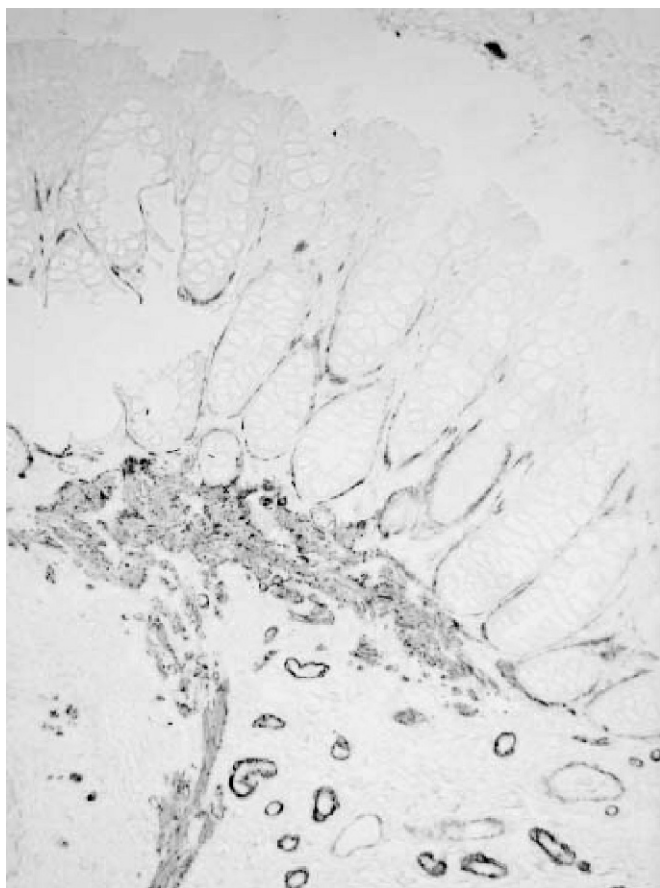


FIGURE 6. CG1 (anti-hTM1) shows no reaction to colonic epithelial cells or to the small intestine. However, strong reactivity was noted with basement membrane of colonic glands, muscularis mucosa, and blood vessel wall. Strong reactivity with smooth muscle also was noted (not shown).

whereas smooth muscle contains mainly hTM1-3. The β -TM gene coding for hTM1 in fibroblast also encodes the β -isoform of smooth muscle tropomyosin (hTMsm β), and both hTM1 and hTMsm β have the identical amino acid sequence.¹ Thus, CG1 antibody recognizes both hTM1 and hTMsm β . Similarly, hTM2 and hTM3 are encoded from the same α -TM gene for another smooth muscle TM form (hTMsm α).¹ CG β 6 likely reacts with hTM2, hTM3, and hTMsm α . Because CG β 6 cannot distinguish between hTM2 and hTM3, it is unclear whether smooth muscle contains both or only 1 of these 2 isoforms.

TMs can induce significant autoimmune responses.¹⁴ Physicochemical and biochemical analyses of 109 human autoantigens showed that several sequences of TM residues were among the most potent autoantigens.¹⁴ TMs also have been found to be the major allergen related to seafood and dust mite.^{26,27} TMs are mostly intracellular proteins, but they also have been found in the cell membrane such as in erythrocytes.²⁸

In the human colon epithelium, the major isoforms of hTM are hTM5 and hTM4.⁸ We have recently demonstrated that hTM5, and not any other hTM isoforms, is externalized in colonic epithelial cells.²⁹ Such an externalization of hTM5 seems to be organ specific and was not seen in the small intestinal enterocytes, although small intestinal enterocytes contain intracellular hTM5.^{8,29} This is intriguing and may be relevant in UC both for effector immune stimulation and for the binding of the autoantibody to colonocytes. Such an organ-specific antigenic display may play an important role for a local trigger of immune response and perpetuation of the disease in UC restricted to the colon and not in the small intestine.²⁹ We believe that this externalization of hTM-5 occurs as a passive transport with another secretory protein called colon epithelial protein (CEP).²⁹ CEP is detected by the mAb 7E₁₂H₁₂ (also known as mAb Das-1) present in colon epithelium, with major localization in the membrane domain, but it is not present in the small intestinal enterocytes.³⁰ It appears that CEP may function as a chaperone for the transport of hTM5.²⁹ The lack of CEP in the small intestine may explain why hTM5 is not externalized by small intestinal enterocytes, which may explain why UC is restricted to the colon. In the gastrointestinal tract, CEP is expressed only in colon epithelial cells.³⁰ However, at extracolonic sites, CEP is expressed in the skin and biliary epithelium,³¹ ciliary epithelium in the eye, and chondrocytes,³² the organs commonly involved in inflammatory bowel disease. As shown in the present study, hTM5 also is expressed at these extraintestinal sites where CEP expression was previously reported.^{31,32} Similar to CEP expression,³¹ hTM5 expression in the biliary epithelium was detected in extrahepatic bile duct, gallbladder, and intrahepatic large bile



FIGURE 7. LC-24 (anti-hTM4) staining of the colon shows minimal epithelial reactivity but strong reactivity of lamina propria cells. Similar stainings also are seen in the small intestine.

ducts, but not in the bile canaliculi and hepatocytes. It is intriguing because primary sclerosing cholangitis associated with UC most frequently involves the extrahepatic and intrahepatic large bile duct system.^{33,34}

hTM5-related immune response may be pathogenetically important because of the surface expression of the hTM5 isoform in colon epithelium.²⁹ Indeed, we reported earlier a predominant autoantibody response against hTM5 locally in the colonic mucosa in >90% of patients with UC.¹¹ Anti-hTM5 isoform autoantibody may bind with colonic epithelium, causing activation of complement system. Halstensen et al³⁵ reported that in UC anti-p40 (anti-tropomyosin) autoantibody of the IgG1 subclass was bound with colonic epithelium, along with activated complement system.³⁵ Such an autoantibody response and complement activation was not found in Crohn's disease.³⁶ A study from Italy reported that about two thirds of the patients with UC but not CD have circulating IgG autoantibody against hTM5.⁴ An anti-TM circulating antibody response in UC but not in CD also was reported in Japanese patients.⁷ Increased expression of hTM5 was noted in the ileal epithelium of the pouch in patients with UC following total proctocolectomy and J-pouch surgery.³⁷ Using immunotransblot assay, we also reported increased expression of hTM5 in UC colon mucosa compared with normal colon.³⁸ The hTM5 expression score correlated to the pouchitis disease activity index ($P < 0.01$). In the absence of pouchitis, the ileal enterocytes showed minimal to no expression of hTM5.³⁷

It is currently unknown whether, like colon epithelium, hTM5 is an autoimmune target for immune effector cells related to the pathogenesis of extraintestinal complications in UC. However, in a study from Japan, significant autoantibody response against TM was demonstrated in 100% of the patients with primary sclerosing cholangitis but not in primary biliary cirrhosis.⁷ Such studies are not yet performed in patients with UC who also have other extraintestinal manifestations such as uveitis, pyoderma gangrenosum, and arthritis.

In summary, the present study shows that the epithelium in the colon and extraintestinal organs commonly involved in UC show predominant expression of the hTM5 isoform, which appears to be an autoantigenic target protein in UC. Such a localization of hTM5 may allow its interaction with effector immune cells and may have significance in the immunopathogenesis of UC and the extraintestinal manifestations associated with UC.

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