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Expression of newly identified secretory CEACAM1[a] isoforms in the intestinal epithelium

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ABSTRACT

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) regulates intestinal immunological homeostasis. However, precise expression patterns of CEACAM1 isoforms remain poorly understood in the intestinal epithelia. Focusing on the small intestinal epithelium of BALB/c mice, we identified three novel splice variants encoding CEACAM1[a]-2, -2C1, and -4C1 by RT-PCR. CEACAM1[a]-2, -2C1, and -4C1 demonstrated secretory properties by transfection experiments *in vitro*. Among them, CEACAM1[a]-4C1 was the major secreted isoform *in vivo* due to the soluble/secreted CEACAM1[a] with a frameshift sequence in the C-terminus, specific for CEACAM1[a]-2C1 and -4C1. CEACAM1[a]-4C1 was capable of binding murine hepatitis virus (MHV) and was detected at approximately 120 kDa in the small intestinal secretions. Neutralizing effects of the soluble CEACAM1[a] on MHV infectivity *in vitro* were demonstrated by using recombinant CEACAM1[a]-4C1. Our data suggest an intrinsic mechanism operated by free CEACAM1 for surveillance of pathogens and maintenance of homeostasis in the intestine.

Introduction

Recognizing that the intestinal epithelium plays a pivotal role in the creation and maintenance of appropriate intestinal homeostasis [1], researchers have focused on identifying the immunological molecules expressed on the apical surfaces of the epithelia that are involved in receiving and transmitting suitable bioactive signals for the induction and regulation of intestinal immune responses.

Recently, carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a member of the CEA family belonging to the Ig superfamily, has been considered as a candidate molecule regulating immunological homeostasis in the intestine [2]. Indeed, dominant expression of CEACAM1 is observed in the intestinal epithelia of humans [3], mice [4] and rats [5]. Furthermore, CEACAM1 is known to serve as a receptor for some bacteria and viruses [2,6]. CEACAM1 includes the membrane-bound isoforms that are generally classified into two groups (CEACAM1-L and CEACAM1-S) referring to the sequences in the cytoplasmic domain: CEACAM1-L contains two immunoreceptor-tyrosine based inhibitory motifs (ITIMs) in the long cytoplasmic domain that generally transmit inhibitory signals [6], while CEACAM1-S lacks ITIM sequences in the short cytoplasmic domain but can send signals to trigger actin cytoskeleton reorganization [7]. In short, the differing signals sent by CEACAM1-L and CEACAM1-S control distinctive biological responses in a wide variety of cell types through *cis*- or *trans*-homophilic binding or heterophilic binding for microorganisms [6].

In addition to the membrane-bound receptor types of CEACAM1, secretory isoforms of CEACAM1 have been characterized with substantial alternative splicing in humans [8] and rats [9]. Some reports have shown the contribution of soluble CEACAM1 in physiological or immunological events [10–12]. However, little is known about the functions of soluble CEACAM1 in the intestine of any animals.

Since CEACAM1 is an obvious orthologue among mammalian species [13], mice have been used as an experimental animal model for the investigation of CEACAM1 functions. However, four splice variants encoding the receptor isoforms but no splice variants encoding the secretory isoforms have been characterized in mice [14], previous studies using mice may have overlooked the possibility of additional isoforms when considering CEACAM1 functions.

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Therefore, in this study, we aimed at elucidating the precise expression profiles of CEACAM1 in the murine gastrointestinal epithelium.

Materials and methods

Mice. BALB/c and SJL/J mice were purchased from SLC (Shizuoka, Japan) and Charles River Japan (Yokohama, Japan), respectively. *Ceacam1*$^{a-/-}$ mice (BALB/c background) were generated as described previously [15]. All animal experiments were conducted in accordance with the guidelines provided by the Animal Care and Use Committee of The University of Tokyo.

Preparation of small intestinal secretions. Luminal secretions of the small intestine were obtained by washing with cold PBS containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Insoluble material was separated from the intestinal wash by centrifuging at 15,000*g* for 20 min. Supernatants were concentrated using a 10 kDa ultrafiltration membrane, and then protein concentrations were determined using a BCA protein assay Kit (Pierce Biotechnology, Rockford, IL). Samples were stored at −80 °C until use.

RT-PCR and sequencing for the small intestinal epithelium samples. Small intestinal epithelia were dissociated from the small intestinal segments as described previously [16]. Total RNA was isolated from the dissociated epithelial cells using a High Pure RNA Tissue Kit (Roche Diagnostics), and mRNA was reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) with oligo dT primer. The cDNA product was used as a template in RT-PCR with a suitable primer set (Table 1 and Fig. 1A). Some PCR products were purified, subcloned into a pGEM-T Easy plasmid (Promega, Madison, WI), and sequenced.

Preparation of CEACAM1^{a}*-expressing transfectants.* The cDNA fragments containing a full-length open reading frame encoding individual CEACAM1^{a} isoform were inserted in the expression plasmid pcDNA3.1(+) (Invitrogen). These recombinant plasmids were transfected into BHK cells by a liposome TransFast Transfection Reagent (Promega). Cells were cultured in DMEM containing 5% FBS at 37 °C.

Generation of polyclonal antibody specific for the frameshift domain of CEACAM1^{a}*-2C1 and -4C1.* In order to generate antibodies to the frameshift domain of CEACAM1^{a}-2C1 and -4C1, a New Zealand White rabbit was injected with a keyhole limpet hemocyanin-coupled synthetic peptide whose sequence is TLLTRWMTSHTLS plus an N-terminal cysteine. Frameshift-specific antibody was purified using affinity-chromatography with a peptide-bound gel (NeoMPS, San Diego, CA).

Immunoprecipitation. Some of the cell samples were lysed in lysis buffer (20 mM Tris, 150 mM NaCl, 1% NP-40, 100 μg/ml of DNase I, 50 μg/ml of RNase A, protease inhibitor cocktail, and 1 mg/ml of BSA; pH 7.6) for immunoprecipitation. The lysate was incubated with rabbit polyclonal antibody 2456Ab [17], anti-frameshift antibody (rabbit IgG), or normal rabbit IgG (Sigma–Aldrich, St. Louis, MO), and then the immune complexes were collected with protein A (GE Healthcare, Piscataway, NJ).

Table 1
Sequences of RT-PCR primers.

Primer	Sequence (5′ → 3′)
F1	ATGGAGCTGGCCTCAGCACA
F2	CGGATCTGGCTCCTTCTGAC
F3	AGCGAGATCTCACAGAGCAC
R1	GTCAGAAGGAGCCAGATCCG
R2	GTGCTCTGTGAGATCTCGCT
R3	AGCAGGACAGACGGACAGAT

SDS–PAGE and Western blotting. Samples dissolved in SDS–PAGE reducing buffer (50 mM Tris, 2% SDS, 6% 2-mercaptoethanol, 10% glycerol, and 0.004% bromophenol blue; pH 6.8) were loaded onto 4–20% polyacrylamide gels (Daiichi Pure Chemical, Tokyo, Japan). After electrophoresis, proteins were electro-blotted onto PVDF membranes. 2456Ab [17] or CC1 mAb [18], both of which specifically recognize the N domain of CEACAM1^{a}, were used as a primary antibody and were further incubated with HRP-conjugated goat anti-rabbit IgG (#31460; Pierce, Rockford, IL) or alkaline phosphatase (AP)-conjugated donkey anti-mouse IgG (#717-055-151; Jackson ImmunoResearch Laboratories, West Grove, PA), respectively. Immune complexes with HRP or AP were visualized with a Super-Signal West Dura Extended Duration Substrate (Pierce) or an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, Hercules, CA), respectively.

Virus overlay protein blot assay (VOPBA). VOPBA was performed to examine CEACAM1^{a}-MHV binding using the MHV strain JHMV srr7 and mAbs specific for the S proteins of JHMV on the electro-blotted membrane as described previously [19]. Immune complexes were further reacted with HRP-conjugated rabbit anti-mouse IgG (ALI3404; Biosource, Camarillo, CA), and visualized using an ECL-Plus (GE Healthcare).

Immunohistochemistry. Frozen sections of the small intestine (7 μm) were incubated with anti-frameshift antibody overnight. Immune complexes were further reacted with Cy5-conjugated donkey anti-rabbit IgG (#711-176-152; Jackson ImmunoResearch Laboratories) for 2 h. Finally, the sections were reacted for 2 h with rhodamine-conjugated lectin wheat germ agglutinin (WGA; Vector Laboratories, Burlingame, CA), and for 20 min with DAPI (Sigma–Aldrich). Labeled fluorescence was observed under a confocal laser scanning microscope (TCS SP2; Leica, Wetzlar, Germany).

Viral neutralization assay. CEACAM1^{a}-4C1 cDNA was inserted in the expression plasmid pEF-DEST51 (Invitrogen). The constructed plasmid is designated as pEF-4C1. 293T cells were transfected with pEF-4C1 or control plasmid pEF-BOS [20] as described previously [21] and then cultured in a serum-free condition using a FreeStyle 293 (Invitrogen) at 37 °C for 3 days. Recombinant CEACAM1^{a}-4C1 with 6× His tag (r4C1-His) in the culture supernatants was purified with a BD TALON Metal Affinity Resins (BD Biosciences, San Diego, CA) and then suspended in PBS. The neutralization activity of the culture supernatants and purified r4C1-His for MHV infection to DBT cells was examined using MHV strain JHMV cl-2 as described previously [22].

Results and discussion

Identification of novel CEACAM1 splice variants by RT-PCR

We initially performed RT-PCR for mRNA obtained from the small intestinal epithelium of BALB/c mice that express the *Ceacam1*^{a} allele [23] using a suitable combination of specific primers (Fig. 1A). When F1 and R1 primers were used for the short-tailed isoforms, 1378 and 838 bp of expected fragments were amplified and confirmed by sequencing as CEACAM1^{a}-4S and -2S, respectively (Fig. 1B). In contrast, when F1 and R2 primers were used for the long-tailed isoforms, not only two expected fragments showing 1392 and 852 bp, which were confirmed by sequencing as CEACAM1^{a}-4L and -2L, respectively, but three unexpected fragments showing 1271, 1137 and 731 bp were amplified (Fig. 1C). Although RT-PCR using F1 and R2 primers often amplified other size of DNA fragments, these three unexpected DNA fragments were always amplified and thus sequenced. Interestingly, the 1271, 1137 and 731 bp fragments also encoded CEACAM1^{a}. The 1271 bp fragment, lacking exon 6, named CEACAM1^{a}-4C1 (GenBank accession no. AB236332); the 1137 bp fragment, lacking