International Immunology, Vol. 21, No. 9, pp. 1013–1023
doi:10.1093/intimm/dxp067

A novel immunoregulatory protein in human colostrum, syntenin-1, for promoting the development of IgA-producing cells from cord blood B cells

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Keywords: B cells, cytokines, cell differentiation

Abstract

Human colostrum contains many bioactive factors that must promote the development of intestinal mucosal immunity in infants. Especially, the presence of certain cytokines such as transforming growth factor (TGF)-β or IL-10 has been of great interest for IgA production as a function of mucosal immune response. In the present study, we attempted to investigate whether unidentified factors inducing generation of IgA-producing cells from naive B cells might exist in colostrum. For this purpose, colostrum samples were directly added to a culture consisting of naive B cells and dendritic cells from cord blood and CD40 ligand-transfected L cells, comparing with recombinant IL-10 (rIL-10) and/or rTGF-β. It was noted that most colostrum samples alone were able to induce IgA-secreting cells at higher levels than rIL-10 and/or rTGF-β. IgA-inducing activity of colostrum was abolished by neither anti-neutralizing mAbs against IL-10 nor TGF-β, though partially by anti-IL-6 mAb. We prepared partially purified fractions from both pooled colostrums with and without IgA-inducing activity and comparatively performed quantitative proteomic analysis by two-dimensional difference gel electrophoresis followed by liquid chromatography-mass spectrometry. As a result, syntenin-1 was identified as a candidate for IgA-inducing protein in colostrum. Western blot analysis indicated that levels of syntenin-1 in colostrum samples were correlated with their IgA-inducing activities. Moreover, we demonstrated that recombinant syntenin-1 could induce preferentially IgA production from naive B cells. These results suggest that syntenin-1 serves as one of IgA-inducing factors for B cells.

Introduction

There is ample evidence that, in addition to the essential nutrients, human breast milk contains a variety of bioactive components such as soluble proteins like IgA or lactoferrin, anti-microbial peptides, cytokines, growth factors, nucleotides and immune competent cells, seemingly compensating physiological delay of the immune responses in growing infants (1, 2). This has been supported by many epidemiological and clinical studies that breastfeeding attenuates the susceptibility to infectious illnesses or certain diseases and the development of allergic disorders in infants (3–5). Colostrum is the first milk produced after birth and is particularly rich in non-nutritional compositions (2). Thus, colostrum is often used to search for some milk components, which must be important for facilitating optimal maturation of the immune responses in the early period of human life (1).

IgA is the predominant Ig isotype in mucosal secretions and plays a crucial role in the first defense against pathogens at the mucosal surfaces. How naive B cells in the mucosal tissues differentiate into IgA-producing cells through Ig class-switch process has been steadily understood (6). The IgA class switch appears to be mediated by T-cell-dependent and T-cell-independent pathways. It has been

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Received 3 October 2008, *accepted* 15 June 2009

Transmitting editor: H. Karasuyama

Advance Access publication 10 July 2009

shown that the ligation of CD40–CD40 ligand (CD40L) between B cells and activated T_h cells triggers the Ig class switch (7, 8) and also that certain cytokines appear to be requisite for its completion (9). Among them, transforming growth factor (TGF)-β is considered to be an essential IgA class-switch factor (10, 11). Other cytokines such as IL-2, IL-5, IL-6 or IL-10 may be important for terminal differentiation of naive B cells into IgA-producing cells as well (12–16). These cytokines have been well known to be present in human breast milk (17), implying the involvement for IgA production by infants as a function of mucosal immune response. Regarding the T-cell-independent pathway leading to IgA class switch and production, recent studies have shown that naive B cells can be triggered through production of a proliferation-inducing ligand and a B-cell-activating factor of the tumor necrosis factor (TNF) family by dendritic cells (DCs), macrophages and intestinal epithelial cells (18–20). In the present study, we have identified a novel factor in human colostrum, syntenin-1, as a candidate of IgA-inducing factor for naive B cells. We also showed that recombinant syntenin-1 could induce preferentially IgA production from naive B cells under culture conditions without other known cytokines. The results suggest that syntenin-1 serves as one of IgA-inducing factors for B cells.

Methods

Colostrum samples

Thirty-five mothers with their full-term newborn infants were recruited from Okada Obstetrics Hospital (Toyama, Japan). After the written informed consent was taken, colostrum samples (~10 ml) were collected from the mother within 7 days postpartum and immediately stored at −70°C. For culture use, each sample was thawed, and the fatty layer and cellular elements were removed by centrifugation at 3000 r.p.m. for 30 min at 4°C. Samples were then dialyzed with PBS for 1 day and next RPMI 1640 for 3 days using Spectra/Pro® Membrane with molecular weight cut off 1000 Da (Spectrum Laboratories, Rancho Dominguez, CA, USA) divided into small aliquots and stored at −70°C until the time of use. This study was approved by the Research Ethic Committee of University of Toyama.

Flow cytometry

Anti-CD19-PE, anti-CD34-FITC and anti-CD1a-FITC mAbs were obtained from DAKO A/S (Carpentaria, CA, USA), and goat anti-human IgA-FITC and IgD-FITC Abs from Southern Biotechnology Associates (Birmingham, AL, USA). Flow cytometric analysis was performed using Coulter EPICS® XL-MCL. System II™ software (Version 3) was used for data processing.

Preparation of naive B cells and DC from cord blood

Cord blood samples were obtained from the umbilical cord of full-term newborns after uneventful delivery. Mononuclear cells (MNC) were isolated from cord blood by the Ficoll-Paque density gradient centrifugation. B cells were purified from cord blood MNC by T-cell depletion with E-rosetting followed by magnetic-activated cell sorting (MACS) (Miltenyi Biotec, Auburn, CA, USA). IgD was expressed on >99% of the B cell population as assessed by a flow cytometer, indicating naive B cells. DCs were generated by the cultures of $CD34^+$ cells isolated from cord blood MNC by MACS in the presence of 100 ng ml^{-1} recombinant granulocyte macrophage colony-stimulating factor, 2.5 ng ml^{-1} rTNF-α and 25 ng ml^{-1} recombinant stem cell factor (all from R&D, Minneapolis, MN, USA) as described previously (21). CD1a was expressed on >86% of the DC population after 12–14 days assessed by a flow cytometer.

CD40L culture system

Induction of Ig-secreting cells (SC) from cord blood B cells was carried out in the CD40L culture system as described (21). The cultures were performed in Iscove's modified Dulbecco's medium supplemented with 50 μg ml^{-1} human transferrin, 5 μg ml^{-1} bovine insulin (all from Sigma, St Louis, MO, USA), 5% FCS (Flow Laboratories) and 10 μg ml^{-1} gentamicin (GIBCO, Carlsbad, CA, USA). A combination of 1×10^5 ml^{-1} B cells and 1×10^5 ml^{-1} DC (irradiated at 3400 rad) were cultured in the presence of 3.75×10^4 ml^{-1} irradiated (7500 rad) CD40L-transfected L cells (a gift of Yong-Jun Liu, DNAX, Palo Alto, CA, USA) in a final volume of 200 μl in 96-well flat microtiter plates (Becton Dickinson, Franklin Lakes, NJ, USA). Colostrum (25% vol/vol) alone, recombinant IL-10 (rIL-10) (200 ng ml^{-1}) and/or rTGF-β1 (0.3 ng ml^{-1}) or rIL-6 (0.3 ng ml^{-1}) (all obtained from R&D Systems) were added to the culture. In some experiments, neutralizing mAbs against human cytokines such as IL-10, latency-associated protein TGF-β1, IL-1β or IL-6 (all obtained from R&D systems) were added to the cultures with colostrum. The cultured cells were harvested on the 6th day for evaluation of IgG, IgA and IgM SC as below.

Enzyme-linked immunosorbent spot

IgG, IgA and IgM SC were evaluated by enzyme-linked immunosorbent spot (ELISPOT). Briefly, cultured cells were added to the ELISPOT plate coated with goat $F(ab')_2$ anti-human Ig (H+L) antibody (Southern Biotechnology Associates). After discarding cells, the plates were washed and biotinylated goat $F(ab')_2$ anti-human IgG, anti-human IgA and anti-human IgM antibodies (all from Southern Biotechnology Associates) were used for secondary antibody. The plates were incubated with avidin–biotin–peroxidase complex in PBS (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA), and 3-amino-9-ethylcarbazole substrate (Sigma) was used for development. Spots were counted using computer-aided ELISPOT manual counting system, (ELIPHOTO, Minerva Tech, Tokyo, Japan).

Partial purification of the colostrum factor that induces IgA SC

Pooled colostrum with IgA-inducing activity, termed active samples, was first applied to a Superdex 200, 10/300 GL column (GE Healthcare, Piscataway, NJ, USA) equilibrated with 20 mM ammonium hydrogen carbonate. The column was eluted with the same buffer at a flow rate of 0.4 ml min^{-1} and the concentration of eluted proteins was monitored by UV detector. Twenty-four fractions (1 ml each) were collected and aliquot of each fraction was tested in the B cell culture and ELISPOT assay for IgA-inducing activity as described above. Fractions with IgA-inducing activity were

pooled and applied to a UNO Q1 anion exchange column (Bio-Rad, Hercules, CA, USA) equilibrated with 20 mM ammonium hydrogen carbonate. Then, absorbed proteins were eluted with a linear concentration gradient of 0–1 M NaCl and 45 fractions were collected. Aliquot of each fraction was dialyzed against 20 mM ammonium hydrogen carbonate and was freeze-dried. The fractions were tested for IgA-inducing activity and active fractions were pooled and applied to a second Superdex 200, 10/300 GL column equilibrated with 20 mM ammonium hydrogen carbonate. Eluted factions were tested for activity. The active fractions were pooled and stored at −70°C. Another pooled colostrum without IgA-inducing activity, termed non-active samples, was partially purified by the same procedures. The fractions of the non-active samples in the second gel filtration chromatography (GFC) corresponding to the fractions with IgA-inducing activity of the active sample were also pooled. Eluted fractions (of both the active and non-active samples) were applied to SDS–PAGE and the gels were stained with silver.

Two-dimensional difference gel electrophoresis and protein identification

The procedure has been described in details elsewhere (22). Partially purified proteins in active and non-active samples were labeled with Cy5 and Cy3 in a ratio 100 μg of proteins to 200 pmol of dye, respectively. A pool of the two fractions was labeled with Cy2 as internal standard on each gel. The labeled samples were mixed with equal volume of 2× sample buffer [8M urea, 4% (wt/vol) CHAPS, 20 mg ml^{-1} dithiothreitol (DTT) and 2% (vol/vol) immobilized pH gradient (IPG) buffer] (GE Healthcare). Labeled samples were combined (50 μg each labeled with Cy5, Cy3 and Cy2 per gel) and subjected to iso-electric focusing (IEF) on a MultiPhor II electrophoresis unit (GE Healthcare) using IPG strips (24 cm, pI 3–10, GE Healthcare). IEF was performed for 40KV hr at 20°C in dark condition. The strips were equilibrated for 10 min in 50 mM Tris–HCl pH 8.8, 6 M urea, 30% (vol/vol) glycerol and 2% SDS containing 65 mM DTT and then for 10 min in the same buffer containing 240 mM iodoacetamide. Equilibrated IPG strips were transferred onto 24 × 20 cm, 12% polyacrylamide gels made between low fluorescence glass plates. Strips were overlaid with 0.5% (wt/vol) low melting point agarose in 25 mM Tris-base, 0.1% SDS and 192 mM glycine containing 0.1% bromophenol blue. Gels were run in Ettan DALTTM II system (GE Healthcare) with 3W per gel at 15°C, until the dye front had run off the bottom of the gels. The 2-D gels were scanned directly with a 2920 2D-Master Imager (GE healthcare). Normalization among three Cy dyes was accomplished by adjusting the maximum pixel values to 55 000 counts with changing the exposure time. The image generated was exported as tagged image format (.tif) files for further protein profile analysis by Decyder software (GE Healthcare). The difference in-gel analysis of DeCyder was used to merge the Cy2, Cy3 and Cy5 images for each gel and detect spot boundaries for calculation of normalized spot volumes/protein abundance. A spot matching between gels and statistical analysis was done by the Decyder biological variation analysis (BVA) software (GE Healthcare). For in-gel digestion, spots of interesting proteins were excised from 2-D gels using an automated spot picker (GE Healthcare). The spots were collected in 200 μl of water in 96-well plates. The recovered gel pieces are washed with aqueous 500 mM ammonium bicarbonate and acetonitrile and then incubated with 12.5 ng μl^{-1} trypsin at 30°C for 15 h. The generated peptides were concentrated and mass spectrometry (MS) analysis was carried out by liquid chromatography-mass spectrometry (LC-MS/MS) (22). HPLC (CapLC, Waters, Milford, MA, USA) was coupled with the quadrupole-time of flight micro mass spectrometer (Micromass, Milford, MA, USA). Instrument operation, data acquisition and analysis were performed using MassLynx 3.2 software (Micromass).

Western blot

Proteins were separated on 10–20% SDS–PAGE and transferred onto a polyvinylidene fluoride membrane. The membranes were incubated with rabbit antibodies against human syntenin-1 (H-48) and lactadherin (H-60) obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bound antibody was detected with HRP-conjugated secondary goat anti-rabbit antibody (Biosource International, Carlsbad, CA, USA) and an enhanced chemiluminescence detections system (GE Healthcare).

Preparation of recombinant proteins

To prepare glutathione *S*-transferase (GST)-syntenin-1 and maltose-binding protein (MBP)-lactadherin fusion proteins, entire coding regions of human syntenin-1 (GeneBank accession number; BC013254) and human lactadherin (GeneBank accession number; U58516) were amplified by PCR using complementary DNA (cDNA) library from PBMC. The PCR-generated fragment of syntenin with *Bam*HI and *Not*I restriction sites was subcloned in frame into pGEX-5X-2 vector (GE Healthcare). The fragment of lactadherin with *Xba*I and *Hin*dIII restriction sites was subcloned into pMAL-C2X vector (New England Biolabs, Ipswich, MA, USA). The oligonucleotide primers used for construction of plasmid were as follows: syntenin-1 forward primer, 5′-CGCGGATCCCCTCTCTCTATCCATCTCTCGAAGAC-3′; syntenin-1 reverse primer, 5′-ATAAGAATGCGGCCGCTTAAACCTCAGGAATGGTGTGGTC-3′; lactadherin forward primer, 5′-CTAGTCTAGAATGCCGCGCCCCCGCCTGCTGGCCGCGCTG-3′ and lactadherin reverse primer, 5′-CAGGCAAGCTTCTAACAGCCCAGCAGCTCCAGGCGCAGGGC-3′. *Pfu* polymerase (Stratagene, La Jolla, CA, USA) was used for all the PCR amplification. The identity of subclones was confirmed by semiautomated sequencing on an ABI 310 DNA sequencer (Perkin Elmer Life Science, Waltham, MA, USA). The plasmids pGEX-5X-2/syntenin-1 and pMAL-C2X/lactadherin were transfected to competent BL21 (DE3) *Escherichia coli* cells (Stratagene) according to standard procedures. The *E. coli* cells harboring pGEX-5X-2/syntenin-1 and pMAL-C2X/lactadherin were grown at 37°C in LB media containing 0.1 μg ml^{-1} ampicillin. At a cell density of 0.5 (OD600), protein expression was induced with isopropyl β-D-thiogalactoside (IPTG) for 3 h. The cells expressing GST-syntenin-1 were harvested by centrifugation at 4000 × *g* for 20 min, resuspended in the washing buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, supplemented with the proteinase inhibitors: 3 mM aprotinine, 5 mM benzamidine, 20 mM leupeptine, 3 mM pepstatin, 5 mM 6-aminohexanoic acid and 200 mM phenylmethylsulfonyl fluoride). The cells

were disrupted 20 times for 20 s in an ice bath by Astrason XL-2020 ultrasonic processor (Misonix, Farmingdale, NY, USA). The lysate was centrifuged at 20 000 × *g* for 30 min and GST-syntenin-1 fusion protein in the supernatant was immediately applied to glutathione-Sepharose 4B column (GE Healthcare) equilibrated with the washing buffer. After extensive washing, the GST-syntenin-1 was eluted with the washing buffer containing 10 mM glutathione. In the case of MBP-lactadherin, the cell lysate in the column buffer (20 mM Tris–HCl pH 7.4, 200 mM NaCl and 1 mM EDTA containing above proteinase inhibitors) was applied to amylose resin (New England BioLabs) equilibrated with the column buffer. After extensive washing, MBP-lactadherin was eluted with the column buffer containing 10 mM maltose. The purity of the fusion proteins was confirmed by SDS–PAGE and Coomassie Brilliant Blue staining and western blot using specific antibodies against syntenin-1 and lactadherin.

Semiquantitative reverse transcription–PCR

The messenger RNA (mRNA) was isolated from naive B cells in the CD40L culture system treated with IL-10 (200 ng ml^{-1}) or GST-syntenin-1 (5 μg ml^{-1}) for 6 days using QIAamp RNA Blood Mini (QIAGEN). cDNA was synthesized from total RNA using SuperScript™ III First-Strand Synthesis System (Invitrogen). Semiquantitative reverse transcription (RT)–PCR was performed to evaluate activating-induced cytidine deaminase (AID), which is crucial for class-switch recombination. PCR conditions were: 5 min denaturation at 95°C, amplification of cDNA for 30 cycles, each cycle programmed for denaturation at 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min 10 s and followed by a final extension phase of 15 min at 72°C. The primers for AID were: forward primer 5′-GAGGCAAGAAGACACTCTGG-3′ and reverse primer 5′-GTGACATTCCTGGAAGTTGC-3′. The PCR products were separated on 2% agarose gel electrophoresis.

Statistical analysis

Each experiment was performed three to four times, and data were presented as mean ± SEM. The significance between different culture conditions, we used non-parametric test (Mann–Whitney *U* test), and correlation was tested by Pearson's χ^2 test. Results were considered significant if $P \leqslant 0.05$ ($^{**}P \leqslant 0.01$ and $^{*}P \leqslant 0.05$). Statistical analysis was done using SPSS software.

Results

Colostrum alone can induce the generation of IgA SC from cord blood naive B cells in the CD40L culture system

We employed the CD40L culture system as described (21) to evaluate the generation of IgA SC from cord blood naive B cells. For this, cord blood B cells were cultured in the presence of DC and CD40L-transfected L cells. Colostrum samples alone were added to the B cell culture, and the added effect of each sample was compared with that of rIL-10, which is a well-known potent IgA-inducing factor (21). As expected, the addition of rIL-10 to the culture resulted in the appreciable generation of IgA as well as IgG and IgM SC. We found that most colostrum samples alone were able to induce the generation of IgA SC at levels comparable to and higher than rIL-10 (Fig. 1). In contrast, nearly all colostrum samples induced lower IgG and IgM SC compared with that induced by rIL-10. Next, we prepared the pooled colostrum sample from five donors showing strong IgA-inducing activity, which we called the active sample. The added effect of this pooled active colostrum sample on the generation of IgA, IgM and IgG SC in the same culture was compared with that of rIL-10 and/or rTGF-β1 or rIL-6 (Fig. 2). Although rIL-10 induced IgG, IgA and IgM SC similarly, it was noted that the pooled active colostrum alone preferentially induced the generation of IgA SC. Human colostrum has been observed to contain approximately equal levels of IgA1 and IgA2 (23), which was confirmed for the pooled active colostrum used here. Nevertheless, regarding subclasses of IgA SC, we found that IgA1 SC were mainly induced by the addition of the pooled active colostrum to the cultures. The similar results were obtained in the cultures with rIL-10 (data not shown). Neither rTGF-β1 nor rhIL-6 induced IgA SC, and a combination of IL-10 and TGF-β1 did not show the enhanced generation of IgA SC as well. To examine the possible roles of IL-10, TGF-β, IL-6 or IL-1β for IgA-inducing activity seen in colostrum, we added neutralizing mAbs against cytokines such as IL-10, TGF-β1, IL-6 or IL-1β to the B cell cultures with the pooled active colostrum sample. As shown in Fig. 3, there was no effect of antibodies against IL-10, TGF-β1 or IL-1β on IgA induction by colostrum. However, it was observed that anti-IL-6 mAb partially inhibited IgA induction by colostrum, suggesting that IL-6 might contribute partly to IgA-inducing activity in colostrum. Based on these observations, we suspected the existence of other IgA-inducing factors in colostrum.

Partial purification of a factor that induces IgA SC from colostrum

In preliminary experiments, we found that IgA-inducing factors in colostrum might be proteinaceous, since IgA-inducing activity in colostrum was abolished by

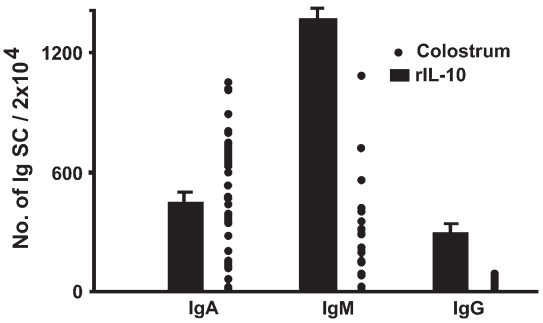

Fig. 1. The influence of colostrum samples on generation of IgA, IgM and IgG SC from cord blood B cells in the CD40L culture system. Thirty-five different colostrum samples (25% vol/vol) or rIL-10 (200 ng ml^{-1}) were added to the B cell culture and each Ig SC was examined on the 6th day by ELISPOT. It was notable that the addition of most colostrum samples to the culture resulted in the predominant generation of IgA SC. In contrast, rIL-10 induced IgA, IgM and IgG similarly. Values in the cultures with rIL-10 are means ± SEMs (n = 3). Results are representative of three separate experiments.

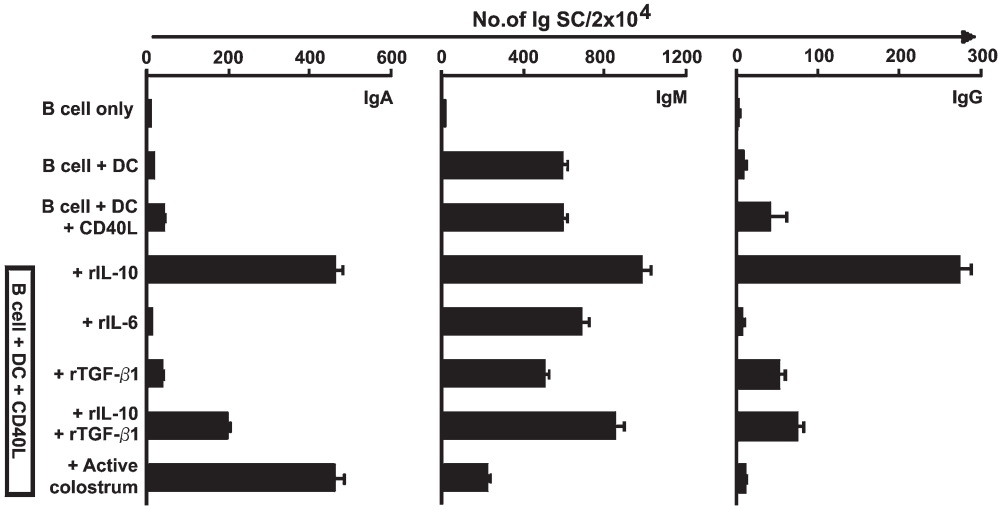

Fig. 2. Preferential IgA induction by the active pooled colostrum. Generation of Ig SC in the CD40L culture system was evaluated as described in the legend of Fig. 1. Cord blood B cells were cultured in different conditions with the active pooled colostrum, rIL-10 and/or rTGF-β1 or rIL-6. Whereas the presence of rIL-10 in culture induced the generation of IgA, IgM and IgG SC similarly, the cultures with the pooled active colostrum sample resulted in the preferential generation of IgA. On the other hand, rIL-6 and rTGF-β1 did not promote IgA production. Values are means ± SEMs (n = 3). Results are representative of three separate experiments.

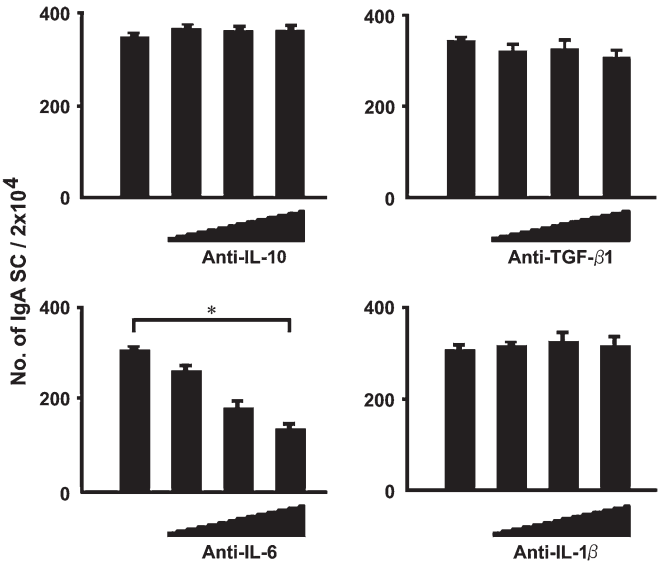

Fig. 3. The added effect of neutralizing mAbs against IL-10, IL-6, TGF-β1 or IL-1β on IgA-inducing activity of colostrum. All cultures were performed in the CD40L culture system with the pooled active colostrum as described in the legend of Fig. 1. The first bar indicates the cultures without neutralizing mAbs, and the other bars represent the added effect of different concentrations of neutralizing antibodies. It was found that anti-IL-10 mAb (0.1, 0.5 and 2.5 μg ml^{-1}), anti-LAP TGF-β1 mAb (0.2, 10 and 50 μg ml^{-1}) or anti-IL-1β mAb (0.2, 0.4 and 0.8 μg ml^{-1}) showed no effect on IgA-inducing activity of colostrum. On the other hand, anti-human IL-6 mAb inhibited partially IgA induction of colostrum in increasing doses (0.2, 0.4 and 0.8 μg ml^{-1}), $*P < 0.05$. Values are means ± SEMs (n = 3). Results are representative of three separate experiments.

pre-treatment with trypsin and chymotrypsin. In addition, the factor was stable through freeze-drying. The pooled active colostrum sample was applied to a GFC. The IgA-inducing activity was eluted as a single peak after a slight delay from the void volume. The molecular mass of the activity was estimated to be ⩾440 kDa, suggesting that it might be associated with macromolecules. The activity recovered from GFC was applied to a UNO Q1 anion exchange column. The activity was absorbed to the resin and eluted with 0.4 M NaCl as a single peak. The activity was applied to a second GFC (Fig. 4A). IgA-inducing activity was eluted as a single peak with molecular mass of ⩾440 kDa (Fig. 4C). The activity was purified 13.6 times and recovery of the activity was 60%. As we found that some colostrums did not contain IgA-inducing activity, we also prepared non-active fractions by the same procedure (Fig. 4B). IgA-inducing activity fraction and the corresponding non-active one were applied to SDS–PAGE and stained with Cy5. The protein pattern of both partially purified fractions was nearly identical (short time exposure to Cy5) and major band of proteins were estimated to be secretory component or lactoferrin (upper band), IgA heavy chain (middle band) and casein or IgA light chain (lower band), respectively, according to their molecular mass and abundance in colostrum (Fig. 4D). The activity seemed to be associated with minor proteins and further purification was required.

Identification of a factor by two-dimensional difference gel electrophoresis followed LC-MS/MS analysis

Partially purified proteins from active and non-active colostrum samples were labeled with different fluorescent dyes and then mixed together and electrophoretically separated in the same 2-D gel. The Cy5 (Fig. 5A) and Cy3 (Fig. 5B) images are corresponding to active and non-active fractions, respectively. Differences in spot population and intensity were visible between two images. Image analysis, i.e. spot detection, within gels and as well as between gels matching with subsequent determination of significant alterations in protein abundances based on normalization by the internal standard was performed by DeCyder BVA. We identified 37

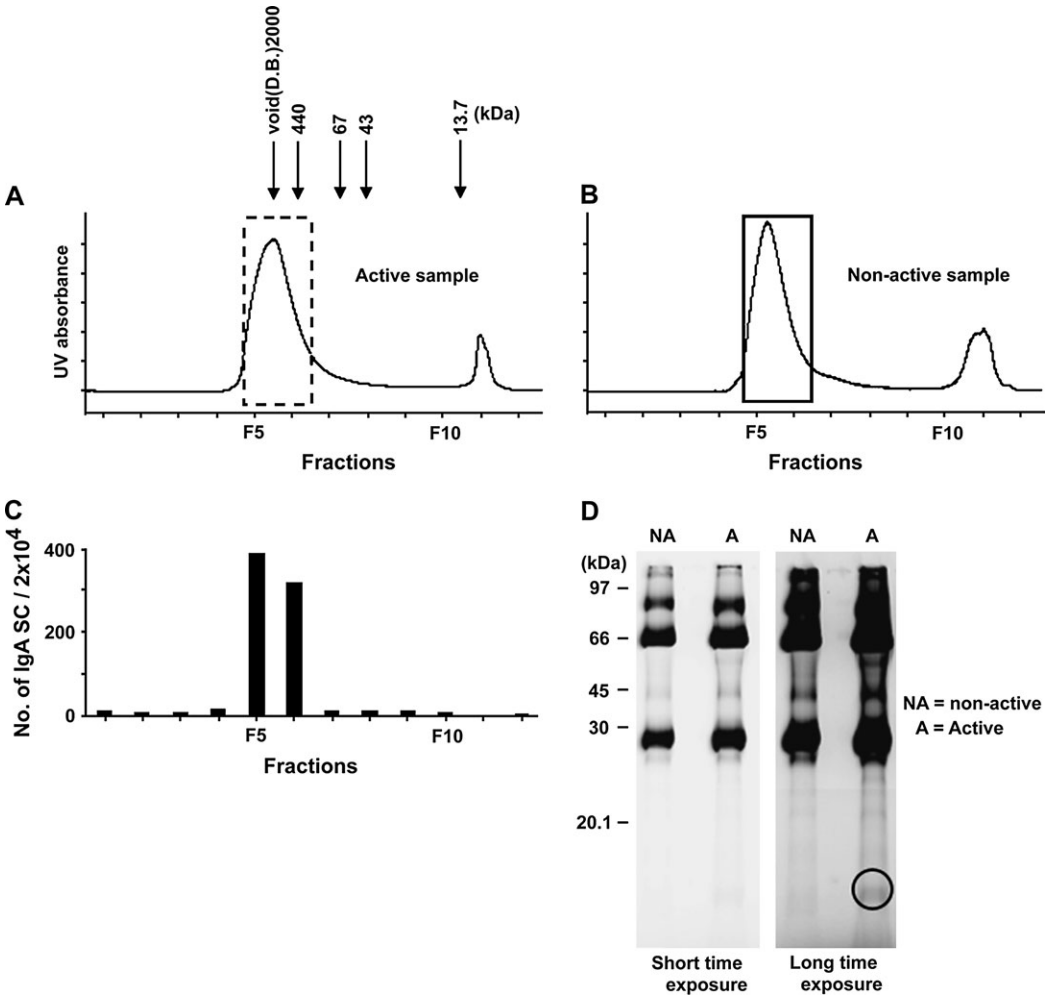

Fig. 4. Second gel filtration column chromatography of active and non-active colostrums. (A) Active fraction and (B) non-active fractions separated by anion exchange chromatography were applied to a second Superdex 200 10/30 GL column and eluted with 20 mM ammonium hydrogen carbonate. Elution of proteins was monitored by UV detector. The arrows indicate the positions where the molecular weight standards were eluted. The standards were blue dextran (2000 kDa), ferritin (440 kDa), albumin (67 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa). (C) IgA-inducing activity in the second GFC in (A) was tested as described in the legend of Fig. 1. The activity was found in fraction F5 and F6. (D) The IgA-inducing fractions (marked with the dashed line) and corresponding non-active fractions (marked with solid line) were pooled. The proteins in aliquots of both fractions were labeled with Cy5 and separated by SDS-PAGE. A and NA denotes the active and the non-active samples, respectively. With long time exposure to Cy5 (right) several minor bands including a low molecular weight band (marked with a circle) were observed selectively in the active sample.

spots significantly increased in active fraction under the conditions where standardized average spot volume ratios exceed two times and P values were $<10^{-2}$ in three parallel gels (Fig. 5A). To take into consideration of post-translational modification and ratio of fluorescence intensity of active spot to corresponding non-active spot, we divided the 37 spots into seven groups. One spot in each group was excised from gels and subjected to gel digestion with trypsin. Resulting peptides then were processed for LC-MS/MS analysis. In a search of the National Center for Biotechnology Information non-redundant database, peptide mass information identified seven proteins (Table 1, positions marked in Fig. 5A). Of the identified proteins, we focused on lactoferrin, lactadherin and syntenin-1. In the case of the small molecular mass protein band (long time exposure to Cy5) marked as a circle (Fig. 4D), protein band was excised and subjected to gel digestion with trypsin. According to MS analysis of the resulting polypeptides, the proteins were identified as Ig kappa, lambda and alpha-1 chain C region.

GST-syntenin-1 fusion protein induces preferentially IgA SC from naive B cells

GST-syntenin-1 fusion protein was expressed in *E. coli* and purified as described in Methods. The fusion protein was expressed upon IPTG induction as a major protein and was exclusively recovered in soluble form. GST-syntenin-1 was added to the B cell culture with different concentrations and also GST alone in the same concentrations. It was demonstrated that GST-syntenin-1 fusion protein was able to induce the generation of IgA SC from cord blood naive B cells in a dose-dependent manner, till saturation at the concentration of 5 μg ml^{-1}, but no IgA induction was detected

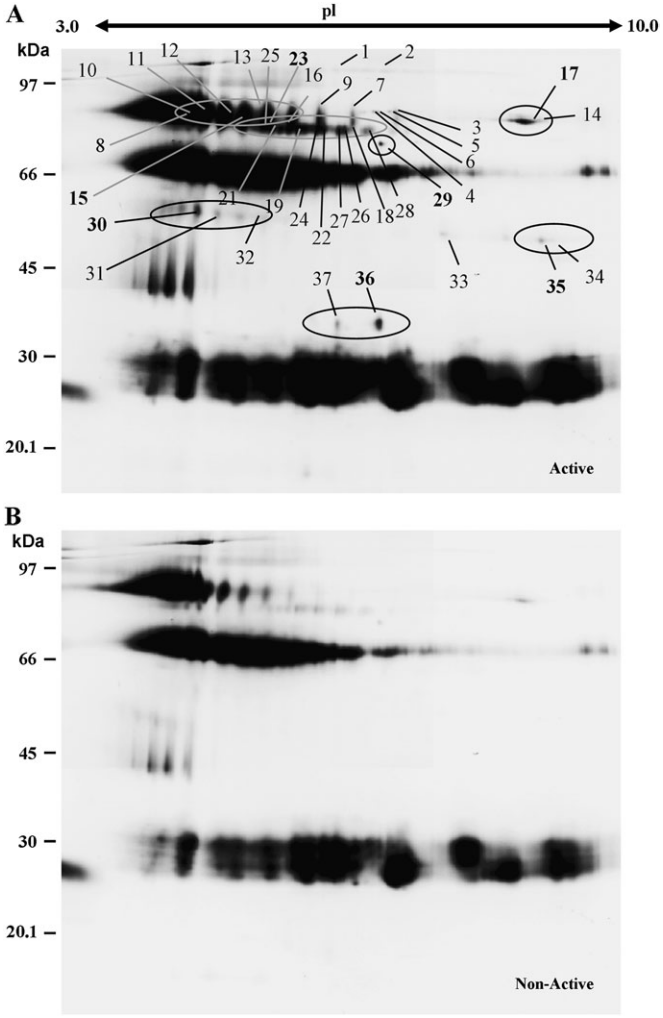

Fig. 5. Differential expressed protein spots displayed in two-dimensional difference gel electrophoresis (2-D DIGE) image. Proteins of active (A) and non-active (B) fractions were labeled with cyanine dyes and mixed together for 2-D DIGE analysis. A pool of the two fractions was also labeled another cyanine dye. The mixture was first separated by IEF (pH 3 to pH 10; horizontal axis) and further separated by 12% SDS–PAGE (vertical axis), which stretches from ~100 (top) to 15 kDa (bottom). (A) Proteins in active fraction labeled by Cy5. (B) Proteins in non-active fraction labeled by Cy3. 2-D gel electrophoresis of the active sample shows 37 spots that are, two times or more, higher in the active colostrum than in the non-active ones. Spots with similar molecular masses and different iso-electric points are grouped as marked by circles. The spots with bold numbers show the identified proteins by LC-MS/MS.

with GST (Fig. 6A). Importantly, the cultures in the presence of GST-syntenin-1 (5 μg ml^{-1}) did not generate IgG SC (Fig. 6B). To confirm the actual IgA induction by syntenin-1, the cells cultured with GST-syntenin-1 were checked for surface expression of IgA on B cells. As shown in Fig. 6(C), it was marked that the sizable number of B cells expressing surface IgA appeared in the cultures with GST-syntenin-1, though less than that with rIL-10. In the case of lactadherin, GST-lactadherin fusion protein expressed in *E. coli* was recovered in soluble fraction. Therefore, MBP-lactadherin was prepared instead of GST-lactadherin. The fusion protein was

Table 1. Proteins identified in partially purified fraction of active colostrum by two-dimensional difference gel electrophoresis followed by LC-MS/MS

Spot no.	Protein	pI	Accession no.	Molecular mass (kDa)	No. of peptides matched	Coverage (%)
15	Polymeric Ig receptor	5.58	gi|31377806	84	14	18
17	Lactoferrin	8.51	P02788	80	13	23
23	Ig mu chain C region	6.35	P01871	50	8	18
29	Complement C3 precursor	6.02	P01024	188	8	5
30	IGHA1 protein	6.21	gi|13543597	55	6	15
35	Lactadherin precursor	8.47	Q08431	43	4	15
36	Syntenin-1	7.05	O00560	32	2	9

Accession numbers are from NCBI or Swiss-Prot.

recovered in a soluble form. However, purified MBP-lactadherin as well as human milk lactoferrin (Sigma) did not generate IgA SC from naive B cells even in the same concentration as that described in human milk (data not shown).

To address whether syntenin-1 is required for the IgA class switching of naive B cells, the expression of AID mRNA was examined by RT–PCR. As shown in Fig. 6(D), AID mRNA was detected even in the control B cells cultured only with DC and CD40L-transfected L cells, although the message was not detected in the cultures of B cells alone (data not shown). This is consistent with the data that ~1.6% of B cells in the CD40L culture system expressed IgA (Fig. 6C). In addition, expression of AID mRNA did not increase by the addition of syntenin-1 or IL-10. These data suggest that syntenin-1 does not trigger IgA class switching but rather acts on post-switched B cells probably as a proliferation or survival factor.

IgA-inducing activity of colostrum is associated with the presence of syntenin-1

To investigate whether syntenin-1 was eluted in other fractions except the IgA-inducing activity fractions, we examined the void volume fractions of the first GFC and the remaining eluted fractions (other than the void volume) of both the active and the non-active samples (Fig. 7A and B) by western blot using anti-syntenin-1-specific antibody. Syntenin-1 (32 kDa) was detected in the void volume fractions (⩾440 kDa) but not in the other eluted fractions. The higher concentration of syntenin-1 in the active sample than that of the non-active one was clearly noticed (Fig. 7C). Finally, we examined 10 colostrum samples for the relation between IgA-inducing activity evaluated by ELISPOT and syntenin-1 levels assessed by western blot (Fig. 8). It was clearly demonstrated that colostrum with IgA-inducing activity contained detectable levels of syntenin-1 but colostrum without IgA-inducing activity did not. The concentration of syntenin-1 in the active colostrums was estimated to be 87.3 ± 8.7 μg ml^{-1} by western blot analysis using recombinant syntenin-1 as a standard (data not shown).

Discussion

The aim of the present study was to identify unknown factors in colostrums regulating generation of IgA-producing cells from naive B cells. For this purpose, we used the CD40L system in which naive B cells were cultured together with DC and CD40L-transfected L cells (21). Using this culture system, we showed that the addition of some colostrum samples alone to the culture resulted in the appreciable generation of IgA SC from cord blood naive B cells (Fig. 1). IL-10 has been shown to promote production of IgA as well as IgG by CD40L-activated naive B cells (14). However, we demonstrated a marked difference between the cultures with colostrum and rIL-10. Namely, the cultures with colostrum alone induced the predominant generation of IgA SC from cord blood B cells, whereas rIL-10 generated IgA and IgG SC similarly (Fig. 2). TGF-β is a well-documented switch factor for IgA (24) and is abundantly present in colostrum. Therefore, it was likely that TGF-β might contribute to IgA-inducing activity seen in colostrum (25). We have observed the association between an increase of serum IgA in newborn infants during one month of age and levels of TGF-β1 and TGF-β2 in colostrum of their mothers (26). Nevertheless, rTGF-β1, even combined with rIL-10, did not generate significantly IgA SC from cord blood naive B cells in our culture system (Fig. 2). These results are contradictory to previously published data on IgA-inducing activity of TGF-β. In contrast to our study, using the almost same culture conditions as ours, Fayette *et al.* (21) have demonstrated that TGF-β can enhance IgA induction by human naive B cells. One possibility for this discrepancy in IgA induction between both studies is the different sources of naive B cells. They used IgD^+ B cells from tonsils as naive B cells, whereas we did cord blood B cells. Nagumo *et al.* (27) have found that the addition of TGF-β to CD40-stimulated cord as well as adult blood B cells were not able to induce IgA secretion even in the presence of IL-10 and IL-2, indicating that the IgA-inducing activity of TGF-β is different on the source of B cells. As for the IgA-inducing activity of syntenin-1, it increased the IgA SC in cord blood B cells (Fig. 6), but syntenin-1 did not have significant effect on adult naive B cells (data not shown). These data indicate that the effect of syntenin-1 could be specific to cord blood B cells.

We prepared the pooled colostrum samples with and without IgA-inducing activity from different donors, which we called active and non-active samples, respectively, and comparatively used them to search for the IgA-inducing factor in colostrum. We identified seven proteins to be higher (two times or more) in the active colostrum than in the non-active one with comparative quantitative proteomic analysis

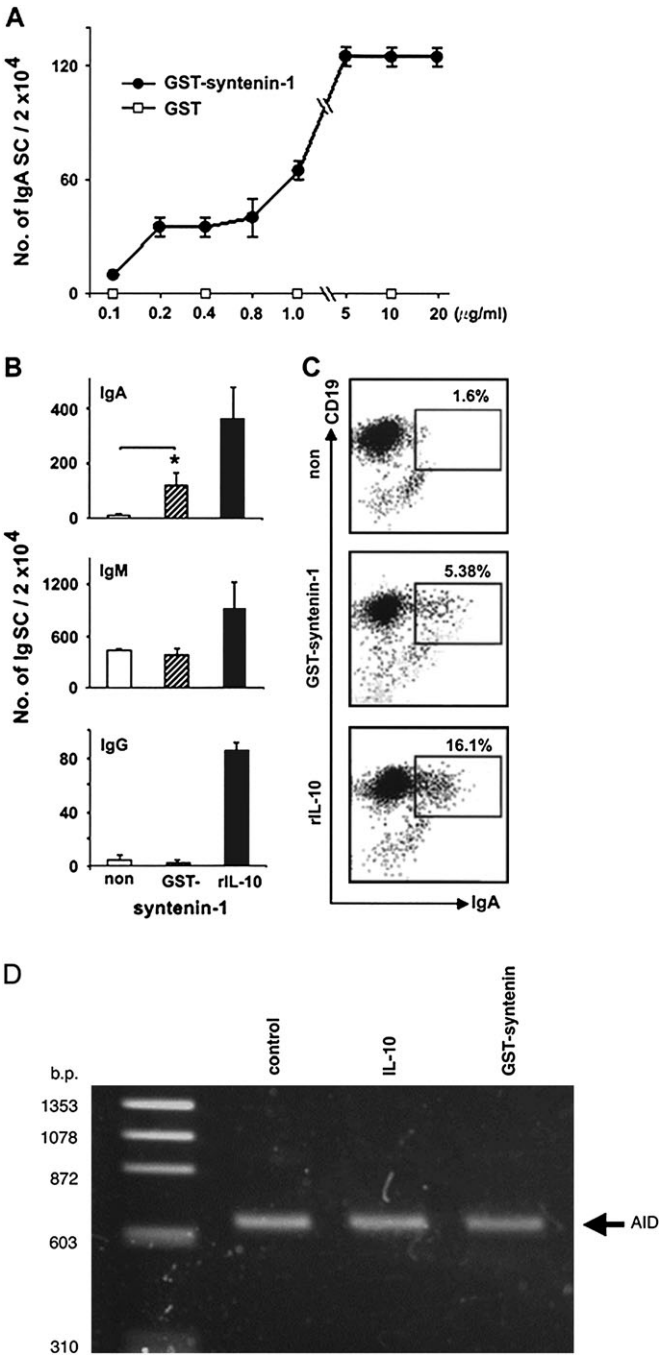

Fig. 6. IgA Induction by GST-syntenin-1. Generation of Ig SC in the CD40L culture system was evaluated as described in the legend of (Fig. 1A and B). (A) GST-syntenin-1 was added to the culture in increasing concentrations and also GST alone in the same concentrations. It was marked that GST-syntenin-1 induced the generation of IgA SC from naive B cells in a dose-dependent manner till saturation at the concentration of 5 μg ml^{-1}, whereas there was no IgA induction by GST alone with any of its concentrations. (B) Whereas rIL-10 (200 ng ml^{-1}) induced IgG, IgA and IgM SC considerably, GST-syntenin-1 (5 μg ml^{-1}) preferentially induced IgA SC. $*P < 0.05$. (C) The cells cultured with GST-syntenin-1 fusion protein 1 (5 μg ml^{-1}) or rIL-10 (200 ng ml^{-1}) were examined for expression of surface IgA. After 5 days, the cells were harvested, stained with both anti-CD19-PE and anti-IgA-FITC and analyzed by a flow cytometer. The sizable number of B cells expressing surface IgA appeared in the cultures with GST-syntenin-1, though less than that with rIL-10. Values in the cultures with rIL-10 are means ± SEMs ($n = 3$). Results are representative of three separate experiments. (D) Evaluation of mRNA for AID by RT–PCR. Naive B cells in the CD40L culture system were cultured with IL-10 (200 ng ml^{-1}) or GST-syntenin-1 (5 μg ml^{-1}) for 6 days. At the end of this time, cells were harvested, RNA extracted, and semi-quantitative RT–PCR was performed to evaluate AID transcript. Control: naive B cells + DC + CD40L cells, rIL-10: naive B cells + DC + CD40L cells + rIL-10, syntenin-1: naive B cells + DC + CD40L cells + GST-syntenin-1.

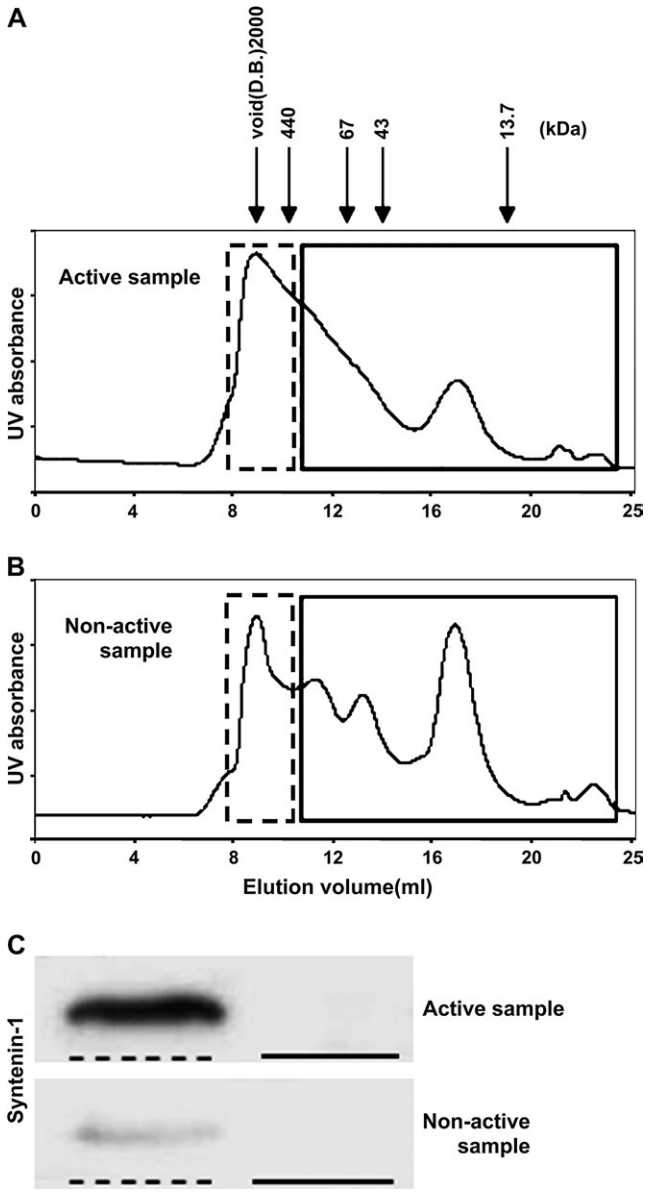

Fig. 7. Elution of syntenin-1 in the fractions with IgA-inducing activity of Superdex 200 10/300 GL column chromatography. (A) Superdex 200 10/300 GL column chromatography of the active colostrum. The dashed and solid squares show the fractions with and without IgA-inducing activity, respectively. (B) Superdex 200 10/300 GL column chromatography of the non-active colostrum. The squares show the corresponding fractions of the active colostrum (A). (C) Syntenin-1 (32 kDa) detected by western blot was found in the IgA-inducing activity fraction (⩾440 kDa) and the corresponding fraction of the non-active sample, whereas no syntenin-1 band could be detected in the proteins eluted in the other fractions. The amount of syntenin-1 in the active sample was much higher than that in the non-active one. Molecular weight markers are the same as in Fig. 4. The figure is representative of three separate experiments.

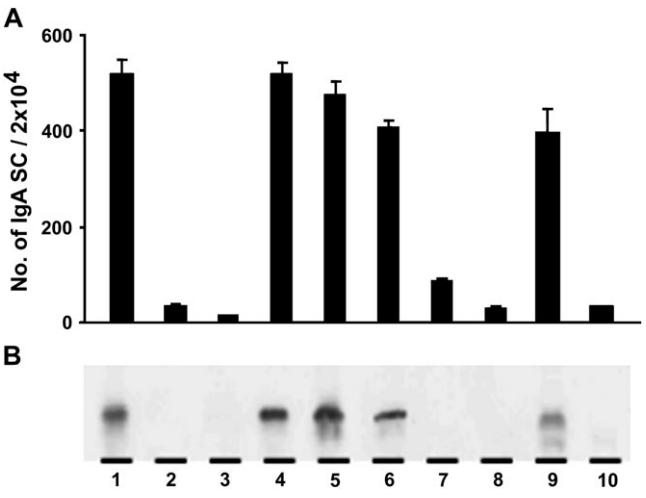

Fig. 8. Association of IgA-inducing activity with syntenin-1 in colostrum samples. Ten different colostrum samples were checked for IgA-inducing activity as described in the legend of Fig. 1. Syntenin-1 was examined by western blot analysis using 10 μl of each sample. Syntenin-1 was detectable only in samples with IgA-inducing activity but not in non-active samples. Values in the cultures are means ± SEMs (n = 3). Results are representative of three separate experiments.

(Table 1 and Fig. 5). Of the identified seven proteins, we focused on lactoferrin, lactadherin and syntenin-1. Bovine lactoferrin has been described to induce both mucosal and systemic immune response in mice where IgA and IgG secretion was enhanced in Peyer's patches and spleen from lactoferrin-fed mice (28). Another study has shown that orally administrated lactoferrin restored humoral immune response in immunocompromised mice (29), and the addition of lactoferrin to the culture medium increased IgA secretion by B cells from mice administered lactoferrin orally (30). Lactadherin binds specifically to rotavirus and inhibits its replication (31) and works as an inhibitor of *E. coli* attachment to intestinal villi *in vitro* (32). Syntenin-1 is known as syndecan-binding protein. Syndecan is proteoglycan expressed on pre-B cells and plasma cells. Interestingly, syntenin-1 is implicated in IL-5 receptor signaling (33), and IL-5 has been described to have a role in IgA-inducing activity (34).

Among these three candidate proteins, we demonstrated that only sytenin-1 might have the ability to induce the generation of IgA SC from naive B cells (Fig. 6). Furthermore, western blot analysis disclosed that syntenin-1 was detectable in colostrum samples with the IgA-inducing activity but not in ones without IgA-inducing activity (Figs 7 and 8). These findings led us to the conclusion that syntenin-1 in human colostrum might be an important factor affecting IgA production. It was shown that IgA induction by colostrum itself was higher than that induced by GST-syntenin-1 fusion protein. In fact, 20 μg ml^{-1} of GST-syntenin-1, which concentration is almost equivalent to those of active colostrums, was less effective to the induction of IgA SC (Figs 6 and 8). So, it is possible that syntenin-1 is not the sole factor in colostrum responsible for IgA-inducing activity, and other factors in colostrum may be implicated with syntenin-1 in IgA induction. Another possibility is that syntenin-1 in human colostrum is in a bound form with other molecules because syntenin-1 (32 kDa) was eluted in a high molecular weight fraction (⩾440 kDa). This binding may affect the activity of syntenin-1 in colostrum to be more active than recombinant GST-syntenin-1 in its produced form. Concerning a role of

syntenin-1 in the process of B cell differentiation, it might be involved in proliferation or survival of post-switched B cells rather than the IgA class switching of naive B cells by the following reasons. AID mRNA have already expressed in the control cells cultured only with DC and CD40L-transfected L cells and GST-synenin-1 did not increase the level of the message (Fig. 6D). On the other hand, the recombinant protein could increase the number of IgA-secreting B cells (Fig. 6C).

Concerning how syntenin-1 might act on naive B cells, it remains unclear whether syntenin-1 could transfer into the cells by endocytic transport. However, we found that syntenin-1 was recognized inside B cells by fluorescence microscopy after 24 h co-culture with cord blood B cells and GST-syntenin-1 (data not shown). It has been shown that B cells can internalize the antigen with BCR-mediated endocytosis (35). In addition, syntenin-1 has been detected in soluble fraction of colostrums and mature milk (36). Therefore, we speculate syntenin-1 is synthesized in mammary epithelial cells, secreted with breast milk and reaches the neonate mucosal immune cells and could be internalized in the cells. Syntenin-1 has also been detected in exosomal fractions of colostrums (37). As exosomes are small microvesicles released from cells and are possible to fuse with plasma membranes and endosomal membranes, syntenin-1 might be more easily released into cytoplasm through the fusion of these membranes *in vivo*.

In conclusion, we propose that syntenin-1 serves as one of IgA-inducing factors for B cells. However, the precise role of syntenin-1 in IgA induction awaits further studies, including animal experiments.

Funding

Grant-in-Aids for Scientific Research, Ministry of Education, Culture, Sports and Technology, Japan (20390294); Ministry of Health, Labour and Welfare, Japan (HMNANCHI03).

Acknowledgements

We thank Dr Masatoshi Okada and the nursing staffs of Okada Obstetrics Hospital for kindly collecting and providing cord blood and colostrum samples, and Hitoshi Moriuchi and Chikako Sakai for excellent technical assistance. We also thank Dr Hidenori Yamanaka (Chemical Evaluation and Research Institute) for his support of quantitative proteomic analysis and Hiroshi Iwamoto (Morinaga Milk Industry Company) for helpful discussion and suggestion. The authors have no financial conflict of interest.

Abbreviations

AID	activating-induced cytidine deaminase
BVA	biological variation analysis
cDNA	complementary DNA
CD40L	CD40 ligand
DC	dendritic cell
DTT	dithiothreitol
ELISPOT	Enzyme-linked immunosorbant spot
GFC	gel filtration chromatography
GST	glutathione S-transferase
IEF	iso-electric focusing
IPG	immobilized pH gradient
IPTG	isopropyl β-D-thiogalactoside
LC-MS/MS	liquid chromatography-mass spectrometry
MACS	magnetic-activated cell sorting
MBP	maltose-binding protein
mRNA	messenger RNA
MNC	mononuclear cells
MS	mass spectrometry
rIL-10	recombinant IL-10
RT	reverse transcription
SC	secreting cells
TGF	transforming growth factor
TNF	tumor necrosis factor

References

1 Lawrence, R. M. and Pane, C. A. 2007. Human breast milk: current concepts of immunology and infectious diseases. *Curr. Probl. Pediatr. Adolesc. Health Care* 37:7.
2 Playford, R. J., Macdonald, C. E. and Johnson, W. S. 2000. Colostrum and milk-derived peptide growth factors for the treatment of gastrointestinal disorders. *Am. J. Clin. Nutr.* 72:5.
3 Goldman, A. S. 2000. Modulation of the gastrointestinal tract of infants by human milk. Interfaces and interactions. An evolutionary perspective. *J. Nutr.* 130:426S.
4 Hawkes, J. S., Bryan, D. L., James, M. J. and Gibson, R. A. 1999. Cytokines (IL-1beta, IL-6, TNF-alpha, TGF-beta1, and TGF-beta2) and prostaglandin E2 in human milk during the first three months postpartum. *Pediatr. Res.* 46:194.
5 Newburg, D. S. 2005. Innate immunity and human milk. *J. Nutr.* 135:1308.
6 Suzuki, K., Ha, S. A., Tsuji, M. and Fagarasan, S. 2007. Intestinal IgA synthesis: a primitive form of adaptive immunity that regulates microbial communities in the gut. *Semin. Immunol.* 19:127.
7 Dubois, B., Vanbervliet, B., Fayette, J. *et al.* 1997. Dendritic cells enhance growth and differentiation of CD40-activated B lymphocytes. *J. Exp. Med.* 185:941.
8 Zan, H., Cerutti, A., Dramitinos, P., Schaffer, A. and Casali, P. 1998. CD40 engagement triggers switching to IgA1 and IgA2 in human B cells through induction of endogenous TGF-beta: evidence for TGF-beta but not IL-10-dependent direct S mu—>S alpha and sequential S mu—>S gamma, S gamma—>S alpha DNA recombination. *J. Immunol.* 161:5217.
9 Litinskiy, M. B., Nardelli, B., Hilbert, D. M. *et al.* 2002. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat. Immunol.* 3:822.
10 Borsutzky, S., Cazac, B. B., Roes, J. and Guzman, C. A. 2004. TGF-beta receptor signaling is critical for mucosal IgA responses. *J. Immunol.* 173:3305.
11 Kehrl, J. H., Roberts, A. B., Wakefield, L. M., Jakowlew, S., Sporn, M. B. and Fauci, A. S. 1986. Transforming growth factor beta is an important immunomodulatory protein for human B lymphocytes. *J. Immunol.* 137:3855.
12 Beagley, K. W., Eldridge, J. H., Kiyono, H. *et al.* 1988. . Recombinant murine IL-5 induces high rate IgA synthesis in cycling IgA-positive Peyer's patch B cells. *J. Immunol.* 141:2035.
13 Bertolini, J. N. and Benson, E. M. 1990. The role of human interleukin-6 in B-cell isotype regulation and differentiation. *Cell. Immunol.* 125:197.
14 Defrance, T., Vanbervliet, B., Briere, F., Durand, I., Rousset, F. and Banchereau, J. 1992. Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *J. Exp. Med.* 175:671.
15 Ramsay, A. J., Husband, A. J., Ramshaw, I. A. *et al.* 1994. The role of interleukin-6 in mucosal IgA antibody responses *in vivo*. *Science* 264:561.
16 Sato, A., Hashiguchi, M., Toda, E., Iwasaki, A., Hachimura, S. and Kaminogawa, S. 2003. CD11b+ Peyer's patch dendritic cells secrete IL-6 and induce IgA secretion from naive B cells. *J. Immunol.* 171:3684.
17 Field, C. J. 2005. The immunological components of human milk and their effect on immune development in infants. *J. Nutr.* 135:1.
18 He, B., Xu, W., Santini, P. A. *et al.* 2007. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. *Immunity* 26:812.

19 Tezuka, H., Abe, Y., Iwata, M. *et al.* 2007. Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. *Nature* 448:929.
20 Macpherson, A. J. and Slack, E. 2007. The functional interactions of commensal bacteria with intestinal secretory IgA. *Curr. Opin. Gastroenterol.* 23:673.
21 Fayette, J., Dubois, B., Vandenabeele, S. *et al.* 1997. Human dendritic cells skew isotype switching of CD40-activated naive B cells towards IgA1 and IgA2. *J. Exp. Med.* 185:1909.
22 Yamanaka, H., Yakabe, Y., Saito, K., Sekijima, M. and Shirai, T. 2007. Quantitative proteomic analysis of rat liver for carcinogenicity prediction in a 28-day repeated dose study. *Proteomics* 7:781.
23 Kiyosawa, I. 1998. Composition of breast milk: proteins. In Kiyosawa, I., ed., *Human Milk in Infant Nutrition*, p. 20. KANEHARA & Co., Ltd., Tokyo.
24 van Vlasselaer, P., Punnonen, J. and de Vries, J. E. 1992. Transforming growth factor-beta directs IgA switching in human B cells. *J. Immunol.* 148:2062.
25 Srivastava, M. D., Srivastava, A., Brouhard, B., Saneto, R., Groh-Wargo, S. and Kubit, J. 1996. Cytokines in human milk. *Res. Commun. Mol. Pathol. Pharmacol.* 93:263.
26 Ogawa, J., Sasahara, A., Yoshida, T. *et al.* 2004. Role of transforming growth factor-beta in breast milk for initiation of IgA production in newborn infants. *Early Hum. Dev.* 77:67.
27 Nagumo, H., Agematsu, K., Kobayashi, N. *et al.* 2002. The different process of class switching and somatic hypermutation; a novel analysis by CD27(-) naive B cells. *Blood* 99:567.
28 Debbabi, H., Dubarry, M., Rautureau, M. and Tome, D. 1998. Bovine lactoferrin induces both mucosal and systemic immune response in mice. *J. Dairy Res.* 65:283.
29 Artym, J., Zimecki, M., Paprocka, M. and Kruzel, M. L. 2003. Orally administered lactoferrin restores humoral immune response in immunocompromised mice. *Immunol. Lett.* 89:9.
30 Sfeir, R. M., Dubarry, M., Boyaka, P. N., Rautureau, M. and Tome, D. 2004. The mode of oral bovine lactoferrin administration influences mucosal and systemic immune responses in mice. *J. Nutr.* 134:403.
31 Newburg, D. S., Peterson, J. A., Ruiz-Palacios, G. M. *et al.* 1998. Role of human-milk lactadherin in protection against symptomatic rotavirus infection. *Lancet* 351:1160.
32 Shahriar, F., Ngeleka, M., Gordon, J. R. and Simko, E. 2006. Identification by mass spectroscopy of F4ac-fimbrial-binding proteins in porcine milk and characterization of lactadherin as an inhibitor of F4ac-positive *Escherichia coli* attachment to intestinal villi *in vitro*. *Dev. Comp. Immunol.* 30:723.
33 Geijsen, N., Uings, I. J., Pals, C. *et al.* 2001. Cytokine-specific transcriptional regulation through an IL-5Ralpha interacting protein. *Science* 293:1136.
34 Harriman, G. R., Kunimoto, D. Y., Elliott, J. F., Paetkau, V. and Strober, W. 1988. The role of IL-5 in IgA B cell differentiation. *J. Immunol.* 140:3033.
35 Rodriguez-Pinto, D. 2005. B cells as antigen presenting cells. *Cell. Immunol.* 238:67.
36 Palmer, D. J., Kelly, V. C., Smit, A. M., Kuy, S., Knight, C. G. and Cooper, G. J. 2006. Human colostrum: identification of minor proteins in the aqueous phase by proteomics. *Proteomics* 6:2208.
37 Admyre, C., Johansson, S. M., Qazi, K. R. *et al.* 2007. Exosomes with immune modulatory features are present in human breast milk. *J. Immunol.* 179:1969.