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Kinetics and Biological Function of Transforming Growth Factor-β Isoforms in Bovine and Human Colostrum

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Abstract Colostrum contains various kinds of cytokines including TGF-β that has potent regulatory effects on cells of the immune system. We compared the levels of TGF-β1 and TGF-β2 in bovine and human colostrum. Based on the isoform-specific ELISA, bovine colostrum collected on day 1 post-delivery retained 53.71±29.55 ng/ml of TGF-β1 and 40.41±21.78 μg/ml of TGF-β2 (n=4), while in human, 381.45± 158.24 ng/ml of TGF-β1 and 41.47±9.63 ng/ml of TGF-β2 (n=5). Thus, dominant TGF-β isoforms were completely opposite between human and bovine colostrum samples. The concentrations of both isoforms declined as lactation proceeded. Biological activities of the colostrum samples were determined using an MV1LU cell line. Consistent with the result from the immunoassay, TGF-β1 in human and TGF-β2 in bovine colostrum were responsible for the antiproliferative activity against MV1LU cells. Furthermore, bovine colostrum increased IgA secretion by LPS-stimulated mesenteric lymph node (MLN) cells, and this effect was abrogated by either anti-TGF-β2 antibody or combined anti-TGF-β1/β2 antibody, but not by anti-TGF-β1 antibody alone. Similarly, TGF-β2 in bovine colostrum enhanced the Ig germ line (GL) promoter activity, which is the earliest event toward IgA isotype switching. Taken together, these results suggest that TGF-β isoforms, differentially expressed in human and bovine colostrum, may promote IgA isotype production in the neonatal intestine.

Key words: Colostrum, TGF-β, human, bovine, IgA

Transforming Growth Factor-β (TGF-β) plays a central role in a broad spectrum of cell functions, including cell growth, differentiation, apoptosis, and migration [5, 18]. Furthermore, TGF-β is involved in embryogenesis, inflammation, regulation of immune response, angiogenesis, wound healing, and extracellular matrix formation [22]. Five isoforms of TGF-β exist and three of them are expressed in mammals and designated TGF-β1, TGF-β2, and TGF-β3 [14]. These three TGF-β isoforms function mutually exclusively *in vivo*, although their actions *in vitro* are quite similar [12]. Different functions of the isoforms are dramatically illustrated by the unique phenotype of mice with disruption of each of the isoform genes: TGF-β1 knockout mice die soon after birth due to a wasting syndrome, accompanied by a multifocal inflammation [11, 28]. In contrast, disruption of the TGF-β2 gene results in cardiovascular defects and impaired lung development, which are not compatible with life after the birth [1, 25]. It was reported that about 60% of TGF-β1 knockout mice die in uterus and 40% appear normal during the first 2 weeks of life but develop a rapid wasting syndrome and die within 4 weeks of age [13]. However, if these TGF-β1 null newborn mice were fed by normal foster mother, they survive like wild-type mice, suggesting that a maternal source of TGF-β is critical for the early development of infant mice [16].

The immune system of newborns is functionally immature and enters a state of extensive differentiation in the early postnatal period. Various cytokines present in maternal milk affect the maturation of the infant's immune system [32]. Many studies have shown that substantial levels of TGF-β1 and TGF-β2 exist in colostrum [23, 24]. In general, TGF-βs are mostly secreted as the latent form [4], and most of TGF-β in bovine and porcine colostrum exist mainly in the latent form [23, 24], whereas 50% of the TGF-β found in human colostrum are in the active form [23, 24]. Thus, although not fully defined, a certain fraction

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of the TGF-β in colostrum appears to be of active form; however, it remains to be precisely determined. On the other hand, it has been demonstrated that the level of TGF-β2 in bovine and human colostrum is higher than that of TGF-β1 [3, 20]. However, another report indicates similar levels of both isoforms in human colostrum [7]. Thus far, there have been no reports to precisely compare the distribution and kinetics of the two TGF-β isoforms between bovine and human colostrum.

In the present study, we first determined the distribution of TGF-β isoforms simultaneously in bovine and human colostrums, and found that TGF-β1 and TGF-β2 were dominant isoforms in human and bovine colostrum, respectively, and that these levels declined as lactation proceeded. These results were further confirmed by a bioassay, using MV1LU cells which are sensitive to TGF-β. In addition, we found that TGF-β present in bovine colostrum increased IgA secretion by intestinal MLN cells.

MATERIALS AND METHODS

Animals

BALB/c mice were purchased from B&K Universal Co. (Fermont, CA, U.S.A.) and maintained *ad libitum* on Purina Laboratory Rodent Chow 5001 (Ralston Purina Co., Richmond, IN, U.S.A.) in an environmentally controlled animal chamber (Myung-Jin Inst. Co., Seoul). Eight to twelve weeks-old mice were used in this study. Animal care was in accordance with the institutional guidelines set forth by Kangwon National University.

Cell Lines and Reagents

A20.3 B lymphoma cell line was provided by Dr. Janet Stavnezer (University of Massachusetts Medical School, Worcester, MA, U.S.A.), and mink lung epithelial-like cell line (MV1LU) was obtained from the American Type Culture Collection (ATCC CCL64, Rockville, MD, U.S.A.). Lipopolysaccharide (LPS) from *Escherichia coli* O127:B8, anti-mouse IgA specific antibodies, and purified mouse IgA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and anti-TGF-β antibodies and purified porcine TGF-β were from R&D Systems (Minneapolis, MN, U.S.A.).

Preparation of Mesenteric Lymph Node Cells and Culture

MLN cells were separated from intestinal fatty tissues by using two forceps in a Petri dish containing PBS. MLN cells were teased and harvested by centrifugation at 500 ×*g* for 5 min. Cells were washed twice with HBSS and suspended in RPMI-1640 medium (Sigma) supplemented with 10% FBS, 50 μM 2-mercaptoethanol, 5 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin.

In the experiment for IgA production, MLN cells (1×10^6 cells/ml) were stimulated with 12.5 μg/ml of LPS and colostrum samples at 37°C for 7 days. Supernatants were then harvested, and the amount of IgA isotype was measured by ELISA [19].

Preparation and Activation of Whey Samples

Bovine colostrum samples were obtained on day 1 to day 5 post-delivery from cows reared at the farm of Kangwon National University. Collected samples were centrifuged at 30,000 ×*g* for 2 h at 4°C to remove lipids. To remove casein, skimmed colostrum was then activated at room temperature (RT) by drop-wise addition of HCl (5 mol/l) to pH 2.0 for 30 min, and then neutralized with NaOH (5 mol/l) to pH 7.0. After activation, the samples were centrifuged again at 30,000 ×*g* and 4°C for 1 h under suction to remove residual lipids.

One- to five-days-old human colostrum samples were obtained from Dankook Medical Center (Chonan, Korea) and prepared as described for the bovine colostrum samples.

ELISA for the Detection of TGF-β

TGF-β1 in colostrum samples was determined by using an improved ELISA, as described before [10]. In brief, anti-mouse-TGF-β1 mAb (1.2 μg/ml) in sodium bicarbonate buffer (pH 9.3) was added to 96-well microplates (Falcon, Becton Dickinson & Co., Oxnard, CA, U.S.A.). Plate was washed with PBST (0.01 M PBS-0.05% Tween 20) followed by overnight incubation at 4°C, and blocked for 1 h with 1% gelatin solution. After washing with PBST, porcine TGF-β1 (standard protein) and colostrum samples were added to each well and incubated for 1 h at 37°C. After washing, anti-TGF-β1 antibody (3 μg/ml) was added to each well and incubated for 1 h at 37°C. HRPO-rabbit anti-chicken-IgG antibody was then added as the secondary antibody for 1 h at 37°C. Plate was washed 3 times, and 0.2 mM 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS, Sigma) was added. Absorbance at 405 nm was measured with an automatic microtiter reader (Bio-Rad 450, Hercules, CA, U.S.A.). In order to determine TGF-β2, anti-mouse TGF-β2 mAb as a coating Ab, anti-TGF-β2 Ab as a detecting Ab, and rabbit anti-goat IgG-HRPO Ab as the secondary Ab were used in this study.

Bioassay of TGF-β Using MV1LU Cells

The biological activity of TGF-β in colostrum was determined by using MV1LU cells [2]. Thus, MV1LU cells (2×10^5 cells/ml) were seeded in 96-well flat-bottom microtiter plates in 100 μl DMEM containing 2% FBS, and they were then incubated overnight. After plates were washed with HBSS, diluted colostrum samples or porcine TGF-β were added in a final volume of 200 μl/well. After 24 h of incubation, [^{3}H]-thymidine (0.5 μCi/ml) was added, and the plates

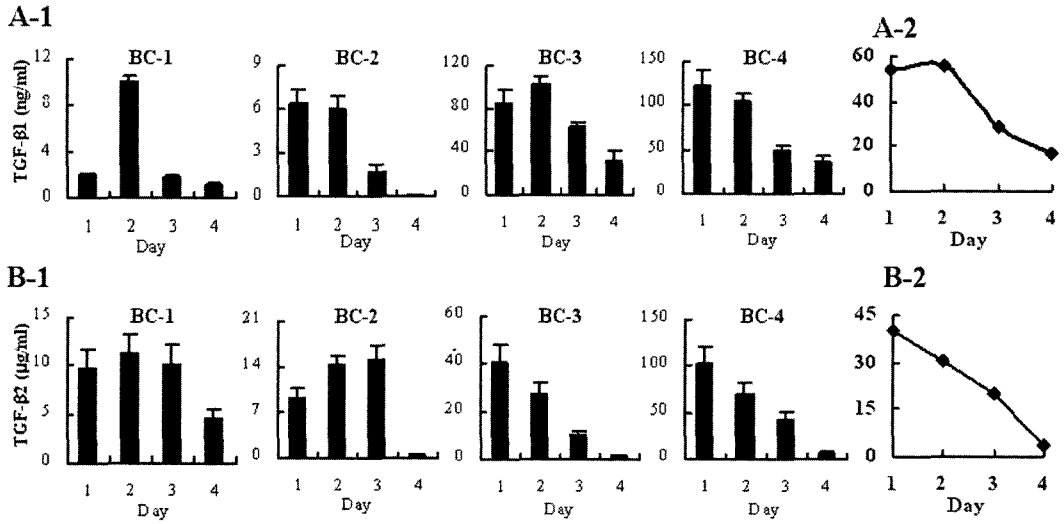

Fig. 1. Detection of TGF-β1 and TGF-β2 in bovine colostrum by ELISA.
Colostrum samples (BC-1- BC-4) were obtained from 4 different cows. Samples were collected up to 3 to 4 days post-delivery. Panel A-1, ELISA for TGF-β1; Panel A-2, Kinetics of TGF-β1 in bovine colostrum sample; Panel B-1, ELISA for TGF-β2; Panel B-2, Kinetics of TGF-β2 in bovine colostrum sample. Data are means±SEM (vertical bars) of six cultures from two separate experiments.

were incubated for an additional 6 h. Cells were harvested onto glass fiber filters, and radioactivity was determined by a liquid scintillation counter (WALLAC 1410, Pharmacia, Finland). In order to distinguish the action of TGF-β1 and TGF-β2 in colostrum, some of the samples were pre-incubated with anti-TGF-β1 antibody (2 μg/ml) or anti-TGF-β2 antibody (0.1 μg/ml) for 30 min at RT before the addition to MV1LU cells.

Transfection and Luciferase Assay

A20.3, a murine B lymphoma cell line, was transfected by an electroporator (Gene Pulser II, Bio-Rad, Hercules, CA, U.S.A.) as described before [26]. In brief, cells (3×10^7) were transfected with 30 mg of GLα reporter [17] and treated with colostrum samples or TGF-β for 16 h. Luciferase activity was determined by using a luminometer (Lmax, Molecular Devices Corporation, CA, U.S.A.), and the transfection efficiency was normalized with β-galactosidase activity.

RESULTS

Dominance of TGF-β Isoform Differs Between Bovine and Human Colostrum

In order to determine the levels of two major isoforms of TGF-β in colostrum, we established two sandwich ELISAs specific to either TGF-β1 or TGF-β2. These two ELISAs at the concentration of 78 pg/ml had no significant cross-reaction to each other (data not shown). Since bovine and human TGF-β isoforms possess identical amino acid sequence [31], we employed the same ELISA to determine each isoform in human and bovine colostrum samples. As shown in Fig. 1A, the concentration of TGF-β1 ranged from 2 to 120 ng/ml and diminished mostly with time, although the concentration varied among the cows. On the other hand, the concentration of TGF-β2 ranged from 10 to 100 μg/ml and also diminished with time (Fig. 1B). Mean values of TGF-β1 and TGF-β2 in 1-day-old colostrum were 53.71± 29.55 ng/ml and 40.41±21.78 μg/ml, respectively. Thus, the bovine colostrum retained much higher level of TGF-β2 than that of TGF-β1.

Since the amounts of TGF-β1 and TGF-β2 in bovine colostrum were significantly different, it was felt of interest to study the distribution of the two isoforms in human colostrum. Similar to bovine colostrum, there were high levels of TGF-β1 and TGF-β2 in one-day-old human colostrum samples, and both levels again diminished with time (Fig. 2). However, the dominant TGF-α isoform in human colostrum was in contrast to bovine colostrums: Mean values of TGF-β1 and TGF-β2 in 1-day-old colostria were 381.45±158.24 ng/ml and 41.47±9.63 ng/ml, respectively, thus human colostrum contain a much higher level of TGF-β1 than that of TGF-β2.

Biological Activity of TGF-β Retained in Colostrum

Based on ELISA, high levels of TGF-β were found in both bovine and human colostrum, therefore, we determined the antiproliferative activity of colostrum using the MV1LU cell line. As shown in Fig. 3A, purified porcine TGF-β1 exhibited slightly higher antiproliferative activity than TGF-β2, but both isoforms showed similar inhibitory pattern. One of the bovine colostrum samples, BC-5D1 (67.2 ng/ml of TGF-β1/574 μg/ml of TGF-β2 as detected by ELISA), showed potent antiproliferative activity (Fig. 3B), whereas

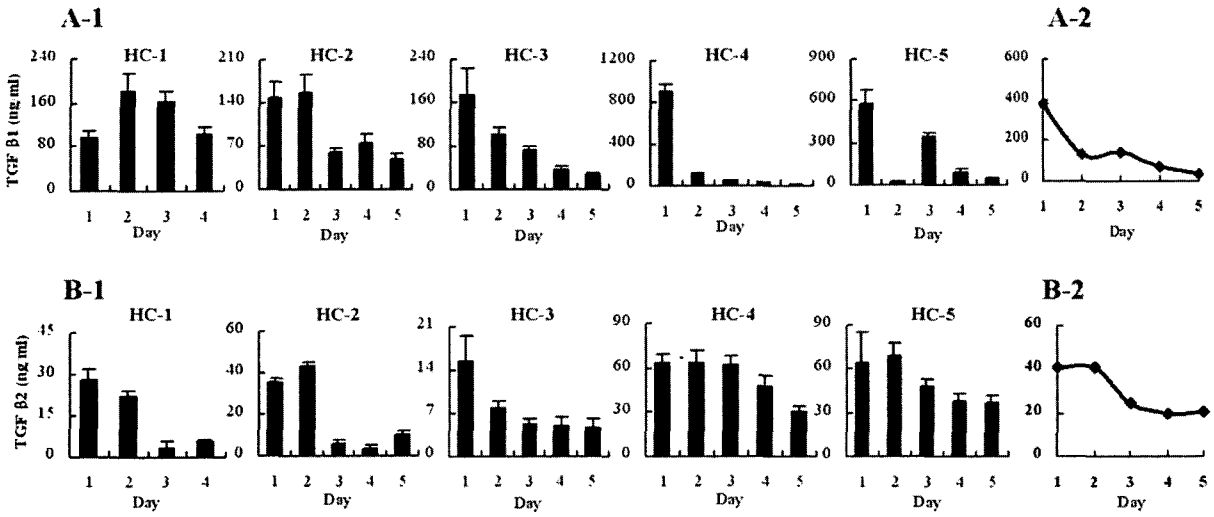

Fig. 2. Detection of TGF-β1 in human colostrum by ELISA.
Colostrum samples (HC-1 - HC-5) were obtained from 5 different individuals. Colostrum samples were collected up to 4–5 days post-delivery. Panel A-1, ELISA for TGF-β1; Panel A-2, Kinetics of TGF-β1 in human colostrum samples; Panel B-1, ELISA for TGF-β2; Panel B-2, Kinetics of TGF-β2 in human colostrum samples. Data are means±SEM (vertical bars) of six cultures from two separate experiments.

BC-2D4 colostrum (0.11 ng/ml of TGF-β1/not detectable TGF-β2) had little effect on the proliferation of MV1LU cells. Considering the dilution factor, we noted that the antiproliferative activity of BC-5D1 sample was approximately 1,000-fold less potent than the purified TGF-β2. One of the reasons for this difference might be due to compositional complexity of the colostrum. Therefore, it was of importance to clarify whether TGF-β is the major cytokine responsible for the antiproliferative effect of BC-5D1 colostrum on MV1LU cells.

To test this possibility, BC-5D1 colostrum was pre-incubated with either anti-TGFβ1 or anti-TGFβ2 antibody before adding to cultures. As shown in Fig. 4A, antiproliferative activity of BC-5D1 colostrum was considerably abrogated by anti-TGFβ2 antibody but not by anti-TGFβ1 antibody, in concordance with the result by ELISA, where TGF-β2 was the dominant isoform in bovine colostrum. Similar to bovine colostrum, we also characterized human colostrums; In contrast to bovine colostrum, antiproliferative activity of HC-4D1 colostrum (917 ng/ml of TGF-β1/63 ng/ml of

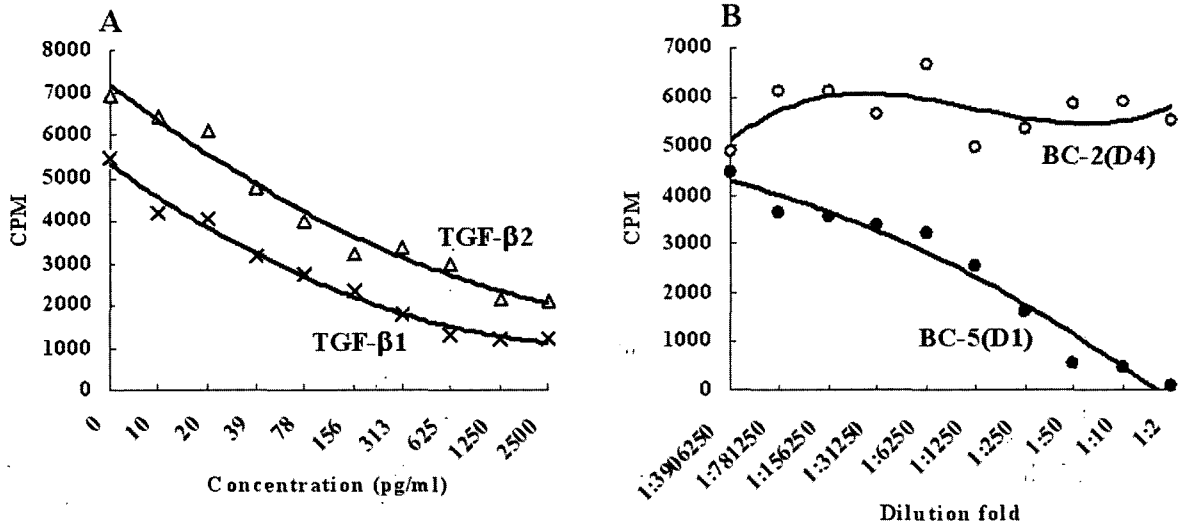

Fig. 3. Antiproliferative activity of TGF-β retained in bovine colostria.
Panel A, Effect of purified TGF-β1 and TGF-β2 on the proliferation of MV1LU cells. **Panel B**, BC-5D1 is a bovine colostrum sample with the greatest concentration of TGF-β, as detected by ELISA (TGF-β1: 67.20 ng/ml, TGF-β2: 574 μg/ml), whereas BC-2D4 is a bovine colostrum sample with the least concentration of TGF-β, as detected by ELISA (TGF-β1: 0.11 ng/ml, TGF-β2: not detectable) among the colostrum samples examined in the present study.

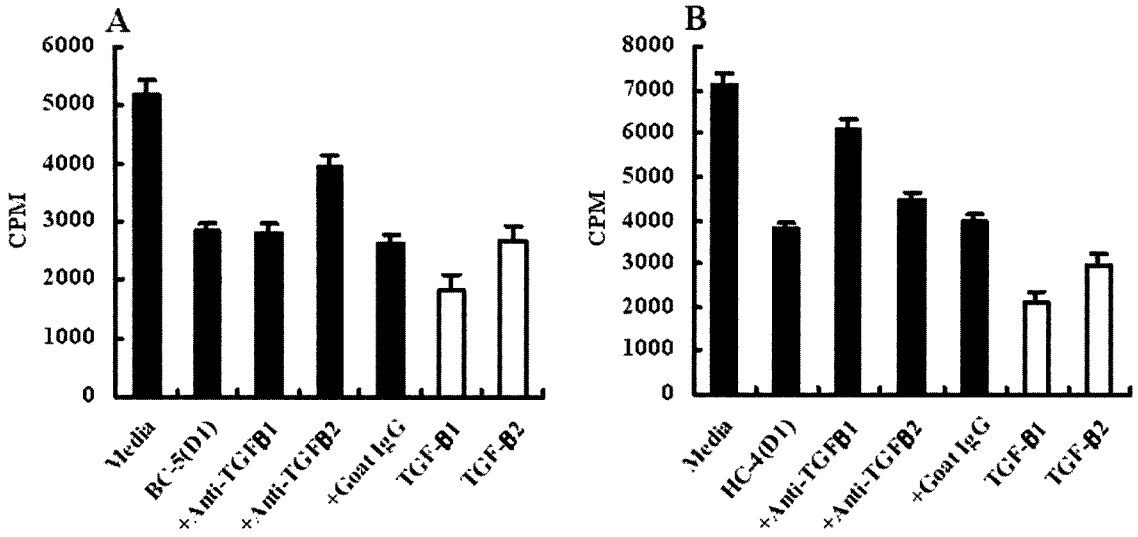

Fig. 4. Anti-TGF-β antibody abrogates the antiproliferative activity of bovine and human colostrum.
MV1LU cells were used to determine the antiproliferative activity of colostrum samples. BC-5D1 is the same bovine colostrum sample used in Fig. 3. HC-4D1 is the human colostrum sample with TGF-β1 (916.9 ng/ml) and TGF-β2 (63.1 ng/ml), as measured by ELISA. BC-5D1 (diluted to 1: 10^4) and HC-4D1 (diluted to 1: 10^3) were pre-incubated with either anti-TGF-β1 (2 μg/ml) or anti-TGF-β2 (0.1 μg/ml) antibody before adding to the cultures. Purified TGF-β1 and TGF-β2 (each 1 ng/ml) were prepared as positive controls. Data are means±SEM (vertical bars) of six cultures from two separate experiments.

TGF-β2 as detected by ELISA) was markedly abrogated by pretreatment with anti-TGFβ1 antibody, but little by anti-TGFβ2 antibody (Fig. 4B). Taken together, these results reveal that TGF-β1 and TGF-β2 are dominant isoforms responsible for the antiproliferative activity of human and bovine colostrum, respectively.

Bovine Colostrum Increases IgA Expression

IgA is the most abundant immunoglobulin isotype in mucosal secretions. It is well known that TGF-β1 selectively induces IgA isotype expression [9, 29, 31]. Thus, it is important to study whether colostrum was involved in the production of intestinal IgA, since maternal colostrum is the first substance introduced into the infant gastrointestinal tract post-delivery. To assess the role of colostrum in the intestinal IgA secretion, mouse mesenteric lymph node (MLN) cells were cultured with the bovine colostrum sample (BC-5D1) and IgA secretion was measured by ELISA (Fig. 5). BC-5D1 colostrum was found to increase IgA secretion by LPS-stimulated MLN cells. This effect was completely abrogated by either anti-TGF-β2 antibody or combined anti-TGF-β1/β2 antibody, but not by anti-TGF-β1 antibody alone. These results indicate that mainly the TGF-β2 retained in the bovine colostrum mediates the enhancement of IgA secretion by MLN cells. The earliest cellular event in Ig isotype switching is the transcription of the corresponding unrearranged constant gene to produce germ-line (GL) transcripts [21]. It was reported that TGF-β1 specifically increases GLα transcripts [6, 15, 27]. In order to gain more evidence that the colostrum can cause IgA isotype switching, we determined the effect of colostrum on GL transcription by measuring the luciferase activity of GLα promoter reporter [17]. As shown in Fig. 6, BC-5D1 colostrum increased the promoter activity up to the level as attained by purified TGF-β1. Similar to the effect on IgA secretion, this effect was also completely abrogated by either anti-TGF-β2 antibody or combined anti-TGF-β1/β2

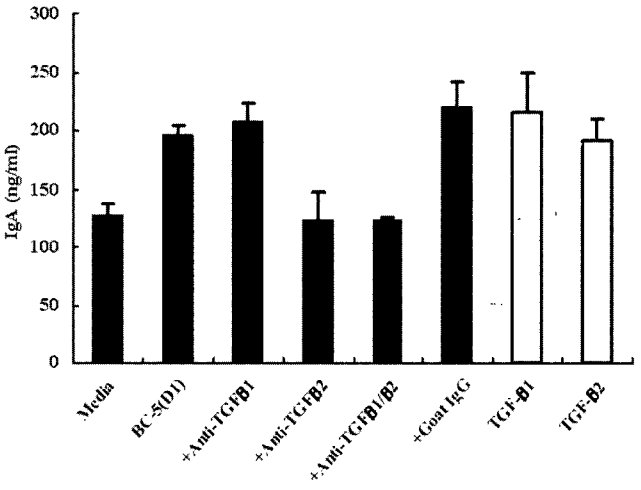

Fig. 5. Effect of bovine colostrum on the IgA secretion by LPS-stimulated mouse MLN cells.
Mouse MLN cells (2×10^5 cells) were stimulated with LPS, BC-5D1 bovine colostrum sample (diluted to 1: 10^4), or purified porcine TGF-β1/β2 (each 1 ng/ml), and they were incubated for 7 days. BC-5D1 (diluted to 1: 10^4) was pre-incubated with anti-TGF-β Ab (anti-TGF-β1 Ab, 2 μg/ml or anti-TGF-β2, 0.1 μg/ml) before adding to the cells as indicated. Production of IgA was determined by IgA isotype-specific ELISA. Data are means±SEM (vertical bars) of six cultures from two separate experiments.

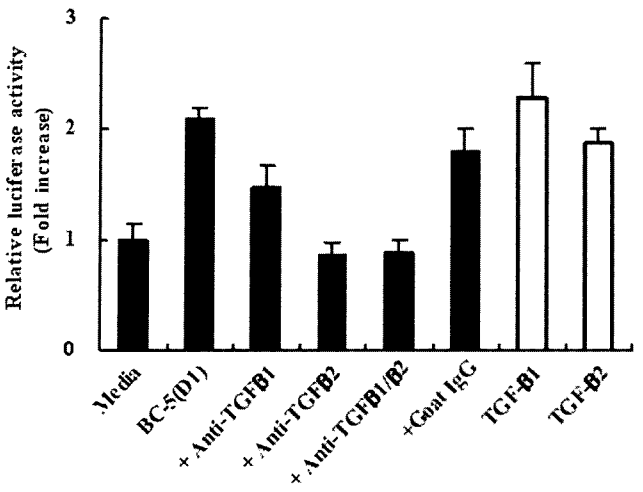

Fig. 6. Bovine colostrum increases GLα promoter reporter activity.

B lymphoma cell line, A20.3 cells, was transfected with GLα reporter (30 μg) and cultured with BC-5D1 colostrum sample (1: 10^4 dilution) or purified porcine TGF-β1 (1 ng/ml). Either anti-TGF-β1 Ab or anti-TGF-β2 Ab or both were pre-incubated with the colostrum sample for 1 h before adding to the cells. Luciferase activity was measured after 16 h of incubation. Data are means±SEM (vertical bars) of six cultures from two separate experiments.

antibody, but not by anti-TGF-β1 antibody alone. These results indicate that TGF-β2, a predominant isoform in bovine colostrum, increases GLα transcription leading to IgA secretion.

DISCUSSION

The present study demonstrates that there are substantial amounts of biologically active TGF-β1 and β2 in colostrum, and that the levels of both isoforms diminish rather quickly with time. Furthermore, dominant isoforms in human and bovine colostrum are TGF-β1 and TGF-β2, respectively. It is well established that both TGF-β isoforms function in a mutually exclusive manner *in vivo*, although their *in vitro* actions are largely overlapping [1, 11, 12, 25, 28]. Consistent with our present results, other investigators have also shown that TGF-β2 is the major TGF-β isoform in bovine colostrum [3, 20]. However, unlike our results, there are no reports to show that TGF-β1 is the major isoform in human colostrum. Instead, it has passingly been described that substantial quantities of TGF-β1 and TGF-β2 are present in human colostrum [7]. In the present report, we did not provide any plausible explanation why dominant TGF-β isoforms differ in bovine and human colostrum. This issue appears to be too complex to be answered and remains to be elucidated. Nonetheless, it seems obvious that TGF-β present in colostrum and milk is critical for the survival of infant, since breastfeeding by normal foster mother was shown to rescue TGF-β1 null newborn mice [16].

During the preparation of colostrum samples in this study, both bovine and human colostrum were transiently adjusted to pH 2.0 to remove casein. It should be noted here that the latent form of TGF-β is known to be activated by this acidic treatment [8]. Therefore, we did not distinguish what fraction of the TGF-β in the colostrum samples was in the active form, and failed to detect any TGF-β activity in the colostrum samples without removing casein. In general, TGF-βs are mostly secreted in latent form [4]. It has been shown that most of TGF-β in bovine and porcine colostrum exists mainly in latent form, while half of the TGF-β found in human colostrum is in an active form [23, 24]. Thus, it appears that some active TGF-βs are present in colostrums, although the quantity varies from species to species. Nevertheless, it is obvious that TGF-β1 and TGF-β2 are dominant isoforms in human and bovine colostrum, respectively, and these levels decline as lactation proceeds. Another important question is "Why do both isoforms decline rather quickly?" Others have also found similar results in bovine, human, and porcine colostrum [3, 20, 24, 33]. Taken together, these observations warrant comment in the context of physiology of infant stomach. As mentioned above, the latent form of TGF-β is easily activated by acidification, which is likely to occur physiologically in the adult stomach with a low pH. However, newborn stomach is not considered to be acidic enough to activate latent TGF-β, but it gradually becomes acidic with time. This may be one of the reasons why newborns acquire high levels of TGF-β from maternal colostrums, particularly at the time of birth. This speculation remains to be elucidated in the future.

A specific role of colostrum-derived TGF-β in the newborn gastrointestinal tract is not understood until now. In this regard, it is highly possible that colostral TGF-β may affect the gut-associated lymphoid tissues leading to the production of IgA which is the predominant Ig isotype in the mucosal surface of the gastrointestinal tract. Our results imply that TGF-β2 present in bovine colostrum contributes to the production of IgA by MLN B cells *in vivo*. Although not yet determined, it is plausible that TGF-β1 is primarily responsible for the IgA induction in the human intestinal tract, since it is the main isoform in human colostrum. Future studies are in need to clarify whether and how these two isoforms are involved in the commitment of IgA B cells during the development of infant intestinal lymphoid tissues.

In summary, a variety of cytokines are provided to newborns through colostrum and milk. Among them, TGF-β is the most potent cytokine in the induction of IgA isotype switching. TGF-β1 and TGF-β2 possess distinctive properties *in vivo* [12]. In the present study, we found that dominant isoforms, TGF-β1 and TGF-β2, differ in human and bovine colostrum, respectively. This information would

be useful in consideration of development of ideal infant formulas.

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