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Transforming growth factor-beta (TGF-β) in human milk

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SUMMARY

The amount of TGF-β contained in human whey was studied by the colony formation of NRK47F cells. It was noted that a factor inducing colony formation did exist in human whey, and its action was neutralized when anti-TGF-β antibodies were introduced. This suggests that TGF-β does exist in human whey. In colostrum, the total amount of TGF-β was 1365·7 ± 242·9 ng/ml, of which the active form comprised 728·1 ± 248·7 ng/ml ($n = 21$). In late milk, the total TGF-β was 952·5 ± 212·6 ng/ml, with an active form of 178·7 ± 157·3 ng/ml. Thus human milk contains a large amount of active TGF-β. Furthermore, it was revealed by the reverse transcriptase polymerase chain reaction that mRNAs coding TGF-β_1 and TGF-β_2 exist in human milk cells. These results suggest that both TGF-β_1 and TGF-β_2 exist in human milk.

Keywords TGF-β human milk RT-PCR

INTRODUCTION

Human milk contains cytokines such as IL-1 [1], IL-6 [2] and colony stimulating factor [3] as well as growth factors such as epidermal growth factor (EGF) [4–6] and TGF-α [7], which are considered to influence somehow the immune system and the growth of neonates. It has been demonstrated recently that bovine milk contains TGF-β_1 and TGF-β_2 [8–10], which play an important role in embryogenesis, inflammation/repair, osteogenesis, and immunoregulation. On the basis of this finding, it is assumed that TGF-β contained in breast milk is somehow involved in the growth of neonates. Human milk is known to contain a factor with TGF-β-like activity [7,11], but how much TGF-β it contains and whether or not it contains active TGF-β is unknown. We studied the amount of TGF-β contained in human colostrum and late milk with bioassay using rat NRK49F cells. We also measured the total amount of TGF-β and the amount of its active form. Furthermore, we extracted RNA from human milk cells and studied the expression of mRNAs of TGF-β_1 and TGF-β_2 with the reverse transcriptase polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Collection of whey

Colostrum was obtained from 21 women, 1–3 days after delivery, and late milk from 24 women 1 month after delivery. The samples were centrifuged at 1700 ***g*** for 10 min to remove lipid. The whey thus obtained was filtered and sterilized using a Millipore filter with a pore size of 0·45 μm and stored at −80°C.

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Assay of TGF-β

One hundred microlitres of a Dulbecco's minimum essential medium (DMEM) medium containing 0·5% Bacto agar (Difco Labs, Detroit, MI) and 10% fetal calf serum (FCS) were poured into each well of a 96-well flat culture dish. This was allowed to solidify at room temperature in order to prepare the bed. The whey was added at 10% or 1% (v/v) to DMEM medium containing 0·33% Bacto agar, 2 ng/ml recombinant human EGF (Wakunaga Pharmaceutical, Hiroshima, Japan), 2×10^4/ml NRK49F cells and 10% FCS, and 100 μl of each whey concentration was dispensed, in triplicate, into the wells. After gelation the plates were incubated at 37°C in 5% CO_2 for 7 days. The number of colonies occurring on the soft agar was then counted. In parallel, 0·1–100 ng of recombinant TGF-β_1 (King Brewing, Kagoshima, Japan) was used, and the number of colonies per well was counted to obtain a standard curve (Fig. 1). The amount of TGF-β was then calculated according to this curve. The numbers of the colonies per well at 10% (v/v) of colostrum and acid-treated late milk were over 200, so we calculated the amount of TGF-β at 1% (v/v) colostrum only. A triplicate assay was always used to measure the TGF-β in the whey.

Acid treatment of whey

Whey was acidified to pH 2 with 1 N HCl for 30 min and reneutralized with 1 N NaOH, and the TGF-β activity in the whey was assayed in the same way as above.

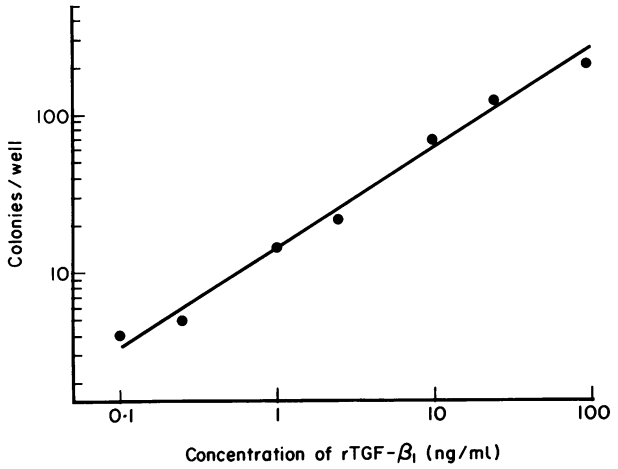

Fig. 1. The number of colonies of NRK47F cells on the soft agar in the presence of recombinant TGF-β_1.

Addition of anti-TGF-β antibody

Rabbit anti-TGF-β IgG (R&D Systems, Minneapolis, MN) was added to the whey of the colostrum, diluted 100-fold, forming a concentration of 10 μg/ml. The colony formation assay was then performed using NRK49F cells. This antibody neutralizes both TGF-β_1 and TGF-β_2.

Primers

Sense (5′-TGACAGCAGGGATAACACACT-3′) and antisense (5′-GTAGGGGCAGGGCCCGAGGCA-3′) TGF-β_1 oligonucleotide primers were synthesized according to the human TGF-β_1 sequence [12] and encompass the base pair regions 1518–1538 and 1828–1808 respectively. The sense (5′-CCCACATCTCCTGCTAATGT-3′) and antisense (5′-GCTGAGTGTCTGAACTCCAT-3′) TGF-β_2 primers were synthesized in accordance with the human TGF-β_2 sequence [13] and the report of Mulheron *et al.* [14], and encompass the base pair regions 1015–1037 and 1259–1240, respectively.

RT-PCR technique

After the colostrum and late milk had been centrifuged at 1700 *g* for 10 min, the cell component was collected. RNA was extracted with RNA Zol B (CINNA/BIOTECX Lab., TX). Four micrograms of RNA were added to a solution containing 50 mM Tris (pH 8·3), 100 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP, 100 pM random hexamer, 20 U reverse transcriptase (Takara Shuzo, Otsu, Japan), and 50 U RNase inhibitor (Takara Shuzo), making a total volume of 20 μl. This was allowed to react at 42°C for 1 h, and was then heat-treated (95°C) for 5 min to produce cDNA. The total volume of 50 μl was prepared from 5 μl cDNA solution, 500 pM primer of each of TGF-β_1 and TGF-β_2 (Fig. 2), 200 μM dNTP, 2·5 U Taq polymerase (Toyobo, Osaka, Japan), and 1 × PCR buffer (Stratagene, CA). This then underwent 35 cycles of amplification. One cycle consisted of 50 s at 94°C, 1 min at 50°C, and 1 min at 72°C. Ten millilitres of the PCR sample were applied to 6% polyacrylamide gels for electrophoresis and then stained with ethidium bromide.

RESULTS

Inhibition of colony formation of NRK47F cells in the presence of human whey by anti-TGF-β antibody

When 1% (v/v) whey of the colostrum was added, a considerable number of colonies were formed (Fig. 3, open column). When 10 μg/ml of rabbit anti-TGF-β IgG were added, the number of colonies decreased greatly (Fig. 3, solid column), indicating that TGF-β is present in colostrum.

Amount of TGF-β in colostrum and late milk

In HCl-treated colostrum, TGF-β was detected at 1365·7 ± 242·9 ng/ml. Even in colostrum which was not treated with HCl, it was detected at 728·1 ± 248·7 ng/ml (Fig. 4). This means that 53·5 ± 16·1% of TGF-β in colostrum was in the active form.

In HCl-treated late milk, the observed TGF-β concentration was 952·5 ± 212·6 ng/ml, which is significantly lower than that in colostrum ($P < 0·01$). In late milk not treated with HCl, the active form of TGF-β was present at 178·7 ± 157·3 ng/ml, which is again significantly lower than that in colostrum ($P < 0·01$). The active form of TGF-β was, therefore, present at 17·9 ± 13·8% in late milk.

Expression of mRNA for TGF-β_1 and TGF-β_2 in cells in colostrum and late milk as measured by the RT-PCR technique

The cells obtained from both colostrum and late milk expressed sequences coding TGF-β_1 mRNA of 310 bp and TGF-β_2 mRNA of 244 bp (Fig. 2). An unknown band, at about 160 bp, was visible in lanes 2, 3 and 4. However, the band coding TGF-β_1 of 310 bp corresponded entirely with that of the positive control, i.e. mononuclear cells which underwent a mixed lymphocyte reaction. When the band of TGF-β_2 was digested with *Hae*III, a fragment of 154 bp and 90 bp occurred, which confirmed the specificity of bands obtained with these PCRs.

DISCUSSION

TGF-β was first identified as a factor which induces the transformation of NRK47F cells [15], normal rat fibroblasts, and promotes their growth in soft agar medium. It has since been demonstrated, however, that TGF-β acts on various cells as a factor inhibiting their growth [16]. TGF-β is present in a latent form, which is not active, and exhibits physiological actions only in its active form after being treated with strong acids or alkalis, etc. [17]. It has been reported that human, bovine and murine milk contains a factor with TGF-β-like action [7,11,18]. It remained unclear, however, as to how much it contains and whether or not an active form of TGF-β with physiological action is present.

It was demonstrated in the present study that a factor which induces colony formation of NRK47F cells in soft agar was present in human whey and that such an action was abolished by anti-TGF-β antibody, which confirmed that the active form of TGF-β is, in fact, present in human milk.

We also measured the total amount of TGF-β by treating the whey with HCl. The total amount was high: 1365·7 ng/ml in colostrum and 728·1 ng/ml in late milk. The active form of TGF-β was present at 53·5% in colostrum and 17·9% in late milk. It has been reported that serum and urine also contain TGF-β [19,20], most of which is in the latent form. The active form of TGF-β has been noted in the synovial fluid of patients with

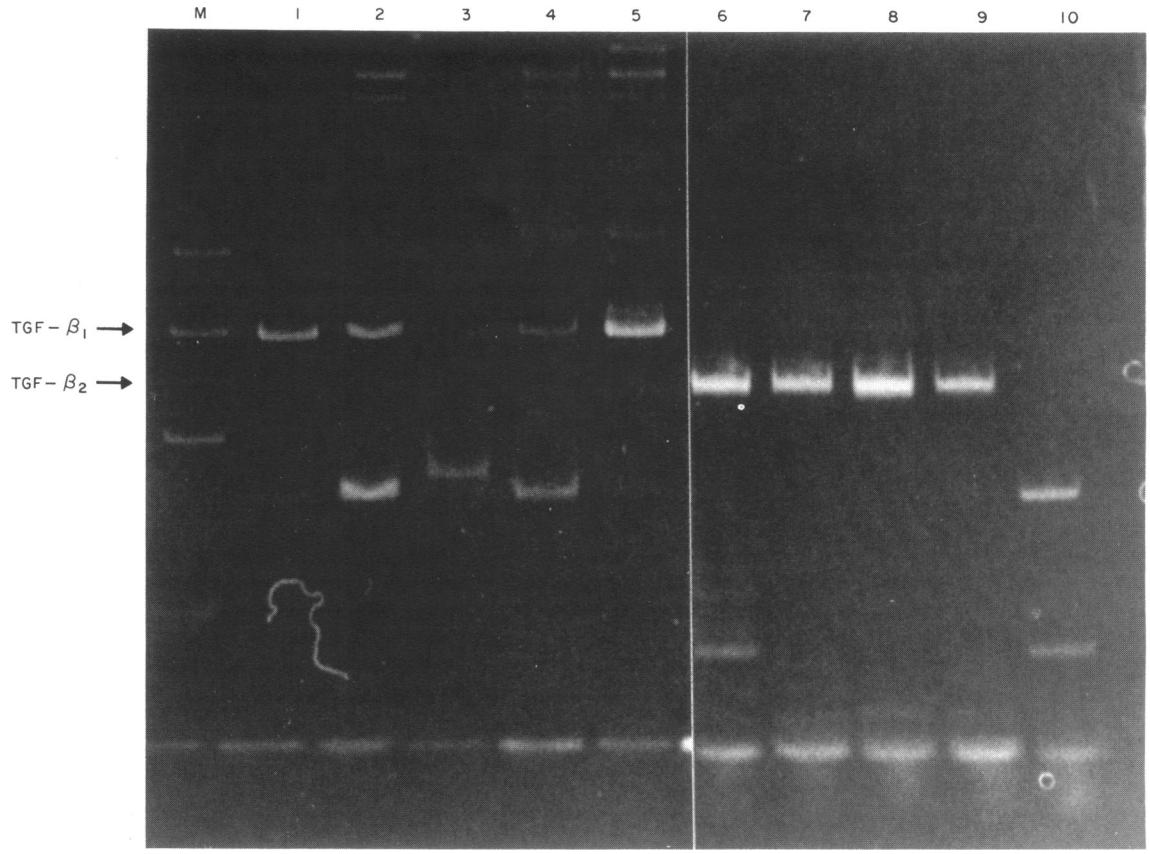

Fig. 2. TGF-β_1 and TGF-β_2 mRNA expression in cells in colostrum (lanes 1, 2, 6 and 7), late milk (lanes 3, 4, 8 and 9) and peripheral blood mononuclear cells stimulated with mixed lymphocyte reaction (lane 5). When verification of the authenticity of the 244-bp TGF-β_2 reverse transcriptase polymerase chain reaction (RT-PCR) product was assessed by digestion with the restriction enzyme *Hae*III, 154-bp and 90-bp bands were revealed (lane 10).

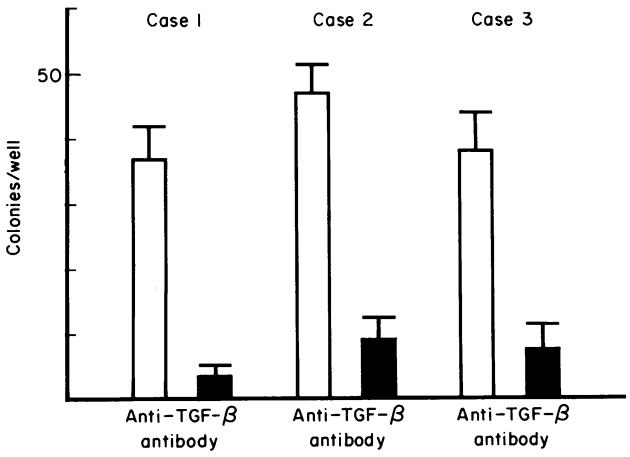

Fig. 3. Blocking of human whey induced colony formation of NRK47F cells by rabbit anti-TGF-β antibody. □, Colony numbers of NRK47F cells in the presence of 1% v/v human whey; ■, colony numbers of NRK47F cells in the presence of 1% v/v human whey and a 1:100 dilution of rabbit anti-TGF-β IgG. Vertical bars show s.d. in triplicate assay.

rheumatoid arthritis, but the amount is only a few ng/ml [21]. It is now clear that both colostrum and late milk contain a very large amount of TGF-β in its active form. Mechanisms for converting the latent form of TGF-β to its active form include activation by sialidase or protease such as plasmin which is secreted from activated macrophages in inflammatory sites, and activation accompanied by acidification of inflammation sites [22,23]. It is well known that a large number of monocytes/macrophages are present in human milk [2]. Protease and sialidase secreted from these cells might be involved in the activation of TGF-β. It has been noted that activated TGF-β acts on surface IgA-negative B lymphocytes and induces a class switch to surface IgA-positive cells [24,25]. This suggests that TGF-β plays a role in the production of IgA in local mammary sites. It has also been reported that activated TGF-β_1 inhibits the growth of the mammary gland and mammary epithelial cells [26–28]. TGF-β also promotes the formation of the extracellular matrix [26,27] or the expression of human milk fat globule epithelial membrane [28]. TGF-β might be implicated in differentiation of mammary cells.

The latent form of TGF-β in colostrum and late milk is bound to be exposed to low pH in the stomach, where it is probably activated. The intestine of neonates is, therefore, exposed to high concentrations of the active form of TGF-β. TGF-β in the intestine appears to act on the production of IgA

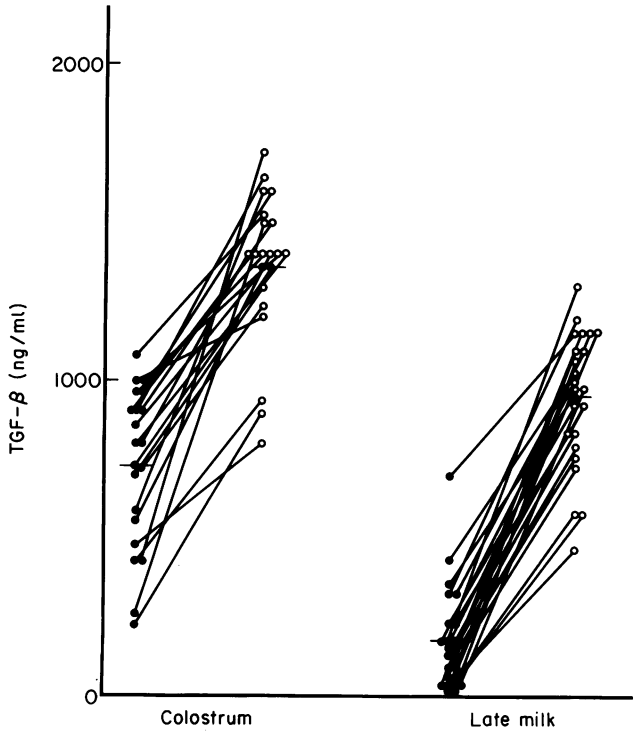

Fig. 4. Amount of TGF-β in colostrum and late milk. ●, Active TGF-β activity in human colostrum and late milk; ○, total TGF-β activity in acid treatment in human colostrum and late milk.

in the intestinal mucosa of infants to serve in defence against infection. TGF-β has been noted to have a potent immunosuppressive action, and bovine milk growth factor, which was found to be identical to TGF-β_1 and TGF-β_2 [9], has been noted to have an inhibitory effect of T cell activation [29]. TGF-β may prevent neonates from being sensitized with food allergens and others. In fact, the incidence of allergy is clearly lower in breast-fed children than in those fed on artificial milk [30].

It is known that there are three isoforms of TGF-β, namely TGF-β_1, TGF-β_2, and TGF-β_3 [16]. In bovine milk TGF-β_1 and TGF-β_2 are present in a ratio of 15:85 [9]. The ratio of the amount of TGF-β_1 and TGF-β_2 in human milk was not investigated in our study. However, when we studied the expression of TGF-β_1 mRNA and TGF-β_2 mRNA in milk cells with the RT-PCR technique, both types of mRNA were detected. This suggests that both TGF-β_1 and TGF-β_2 are present in human milk. It will be interesting to study the mechanisms by which cells produce TGF-β in milk. Milk contains macrophages, neutrophils, lymphocytes, and mammary epithelial cells. It is now necessary to identify TGF-β_1-secreting cells by immunohistostaining or by *in situ* hybridization. It has recently been reported that MCF-7 cells, human mammary cells, produce TGF-β_1 and TGF-β_2, and that TGF-β_2 is produced more predominantly in a ratio of 22:78 [31]. It is possible that TGF-β_2 is also more predominant in human whey, as in the case of cows. Further exploration will be needed to identify cells which produce TGF-β isoforms and elucidate the physiological actions of TGF-β.

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